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MSS. for the successive numbers of Vol. 26 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICES.

WILLIAM DOBINSON HALLIBURTON, 1860–1931.

On May 21st last year the physiological and biochemical worlds were deprived of one of their most distinguished members, King's College of one of its most faithful professors and all who had the privilege of his friendship of one whose loss will be impossible to replace.

Halliburton was born in 1860 and after preliminary education at University College School he followed his natural bent and proceeded to University College and entered as a student in the faculties of Medicine and Science. He obtained the degree of B.Sc. at the age of 19 and his M.D. when only 24. Further study at Vienna equipped him for his future career which may be said to have begun in the year 1883 on his appointment as Sharpey Scholar and subsequently as assistant to Professor (now Sir Edward Sharpey) Schafer.

His first communication on a scientific subject was delivered to the Physiological Society in December 1883, at a meeting held at his own college.

It was not till 1889 that Halliburton moved from University College and in this year he succeeded Gerald Yeo as Professor of Physiology at King's College, a position he held till 1923, when he retired from active academic work owing to a breakdown whilst on holiday during the previous Christmastide.

Further distinctions Halliburton possessed were Fellowship of the Royal Society (1891) and Fellowship of the Royal College of Physicians (1892). He was awarded the honorary degree of LL.D. by the Universities of Toronto and Aberdeen and served as a member of the Council of the Royal Society and had been elected a Fellow of both King's and University Colleges. He was also a Senator of the University of London.

It is easy to detail the bald facts of a career; it is not difficult to give a list of publications; but in the case of Halliburton it is almost impossible to describe the personality in such a way as to convey a just and true impression. A more able pen than the writer's is required.

As a worker Halliburton was untiring; as an author he was prolific. One has but to mention that during his tenure of the chair at King's College some 300 papers emanated from the Department.

The textbooks produced by him tell, by their rapid and frequent editions, the esteem in which they were held by successive generations of students.

His genius for collecting and collating facts is shown in his monumental *Textbook of Chemical Physiology and Pathology*, his first book, published in 1891 and translated into German.

His supreme faculty for clear writing is evidenced by his *Handbook of Physiology*, still perhaps, after some twenty editions, the elementary manual

most widely used by medical students. His *Essentials of Chemical Physiology*, now in its fourteenth edition, is also a favourite book.

Other testimonies to his industry are the valuable *Abstracts of Chemical Physiology* commenced by him and compiled annually for many years for the Chemical Society, and the fact that during the busy period of the late war, while engaged on other matters, he found time to initiate and edit that indispensable Journal *Physiological Abstracts*. For some years not only did he edit this without other help, but in addition did the major part of the actual abstracting.

Other publications include *The Chemical Side of Nervous Activity* (1901), *The Biochemistry of Muscle and Nerve* (1904) and *Physiology and National Needs* (1919).

Halliburton was also a member of numerous boards and committees, positions for which his unruffled and equable temperament, his courtesy and his happy facility of speech admirably fitted him.

He delivered the Goulstonian (1893), the Croonian (1901) and the Oliver-Sharpey (1907) lectures at the Royal College of Physicians.

At King's College, Halliburton threw himself indefatigably into the cause of the Medical Faculty and the Medical Student, not only in the position of Dean, but also as a friend. The affection which his students bore for him was touchingly shown when, in January 1923, they were informed of his illness. No one who was present could avoid being envious of such a friendship between teacher and student.

How few teachers are there now whose illness would be a personal matter to their students and whose retirement would be an individual loss. Yet this was the relationship between Halliburton and his pupils.

As a small token of their affection, on his partial recovery, there was a presentation to him by the students of the Medical Faculty. His attitude to them is shown by his opening words in reply: "Dear boys and girls." He made the students his children, their troubles his affair, their successes his joy.

The writer will refrain from giving examples of his kindness and consideration to those under him. During his life he did not crave publicity for such matters. Now that he is no longer with us we can honour him by respecting his wishes.

As a speaker, Halliburton was outstanding. He brought to King's a scientific knowledge coveted by many and a facility of expression, as exponent or as adversary, coveted by all.

After his retirement from active teaching our Society did him the highest honour in its power. It elected him as its only honorary member. By this we recognise the position Halliburton occupied in the world of Chemical Physiology. While perhaps not the founder of the subject, he is without any doubt responsible, by his own work not less than by his writings, for the firm position now held by Biochemistry among the other sciences.

During his tenure of the chair at King's College, Halliburton collected round

him a brilliant band of fellow-workers. One need only mention for example C. J. (now Sir Charles) Martin, Sir Frederick Mott, W. E. Dixon, O. Rosenheim and F. S. Locke.

Halliburton took an active part in the affairs of the British Association. He was twice president of Section I. Fond of travel, he and Mrs Halliburton were well able to gratify their desires in this direction. As a result their circle of friends was indeed wide and drawn from most of the countries of the world.

In his home in Marylebone Road there was the same order, routine and peacefulness that characterised the man. Here in this oasis from the rush of London one was ever sure of the warmest welcome.

During the years of his forced retirement from active participation in the events of the world, tended with unflagging devotion by his wife who fortunately survives, his only relaxation that of motoring, he nevertheless took the greatest interest in two topics, the revision of his textbooks and the architectural improvements and additions to his old department at King's College.

Since his death still further extensions have been carried out and as a small measure of the esteeem in which we hold his memory these are to be designated the "Halliburton Laboratories."

As an expression of his love for his old scientific home, Halliburton left a munificent bequest for the benefit of the department which he founded and in which he laboured so long, so faithfully and with so much kindliness.

J. A. H.

MORNA MACLEOD, 1906–1931.

Morna Macleod was born on May 27th, 1906, in North Queensland, and spent her early childhood there and at Kalgoorlie, Western Australia, where her father was general manager, for Messrs Bewick, Moreing and Co., of a group of gold mines.

In 1917 she came to London and entered St Margaret's School, Harrow. After matriculating in London University she continued her general education in art and modern languages, including Russian and Spanish, for a further two years before turning to science. She entered University College in 1924 and after gaining an Honours B.Sc. degree in Chemistry and Physics in 1927, spent another year as a research student in the Ralph Forster Laboratories of Organic Chemistry.

During this time she collaborated with Dr Le Fèvre in an investigation of the reaction between ammonium sulphide and formaldehyde solution, and also carried out experiments on the acetylation of diethylene oxide, the results being published in the *Journal of the Chemical Society*.

From 1928 she was attached as a voluntary worker to the Department of Biochemistry of the Lister Institute where she joined in the investigations concerning the physiological significance of phosphoric esters. She was the joint author of papers, published in the *Biochemical Journal*, on the iodimetric estimation of aldoses and on calcification *in vitro*, and, for some time past, had been engaged in studying the hydrolysis of hexosephosphoric esters by bone phosphatase.

Morna Macleod had a very real love for her work, and combined enthusiasm with critical judgment and great perseverance in face of difficulties. She found great interest and pleasure also in music, art and literature and in the normal activities of youth. Her gentle and lovable personality, with its strong vein of dry Scottish humour, endeared her to all her friends and companions, to whom her tragically sudden death on December 6th, 1931, came as a very great blow.

R. R.

XXXI. AN INVESTIGATION INTO THE CAUSES OF DEATH OF SALMON AND SEA TROUT SMOLTS IN THE ESTUARY OF THE RIVER TEES.

By BERNARD ALFRED SOUTHGATE, FREDERICK TOPHAM KEY PENTELOW, and RONALD BASSINDALE.

(*Received January 18th, 1932.*)

Introduction.

The Tees was formerly a noted salmon river, large numbers of both salmon (*Salmo salar* L.) and sea trout (*Salmo trutta* L.) being caught by rods in the upper reaches and in nets in the estuary. For some years past however there has been considerable mortality amongst adult fish whilst attempting to pass through the estuarine reaches on their way to the spawning grounds, and again when descending to the sea as kelts after spawning. In addition, a large proportion of the young salmon and sea trout in the smolt stage are killed during their seaward migration in the months of April, May and June. This mortality is undoubtedly brought about by the heavy pollution to which the estuary is now subjected. The polluting material includes large volumes of sewage and the waste liquors from numerous industrial plants on the estuary banks. The work to be recorded was undertaken to determine the relative importance of these different types of polluting materials in contributing to the toxicity of the estuarine water into which they are discharged.

An account of the physical features and tidal conditions of the estuary has already been published by the Water Pollution Research Board [1931]. The limit of tidal action is some 25 miles from the mouth, salt water penetrating about 15 miles from the sea at high water during spring tides. For a distance of approximately 12 miles from the mouth the estuary channel is navigable and is maintained, by dredging, at a depth of not less than 12 feet at low water. The banks here consist either of artificial training walls, or of wharves and similar structures. It is into this section of the estuary that the bulk of the pollution, both of domestic and industrial origin, is discharged. Above the navigable reaches the channel is undredged and runs between natural or mainly natural banks.

Polluting effluents.

The industries of Tees-side are concerned predominantly with the production of iron and steel and the majority of toxic discharges arise, directly or indirectly, from these processes. Other miscellaneous effluents are, in

comparison with these, of but minor importance and do not contribute appreciably to the toxicity of the estuary water.

The main types of polluting material discharged are:

(1) *Crude sewage.* Crude sewage is discharged from all the towns on the banks of the estuary, the largest being Middlesbrough (pop. 136,000) and Stockton (pop. 67,200); the total contributing population is approximately 275,000. Water carriage of sewage is now employed throughout practically the whole of the area.

(2) *Coke-oven effluents.* Coke-oven effluents are produced in considerable volumes during the manufacture of the large quantities of coke used locally in iron smelting. Two methods are employed for the recovery of ammonia from the coke-oven gas; the direct and semi-direct processes giving rise to two types of effluent:

(A) *Coke-oven effluent, direct ammonia recovery.* In this type of installation the heavy tar is removed from the crude coke-oven gas at a temperature of 65 to 70° before the liquor condenses. The hot gas then passes through a saturator where the ammonia is converted into sulphate. The waste liquor consists of a mixture of the condensate from the water-cooled condensers and the washing and cooling water from the jet condensers. The liquor then flows through settling tanks to remove naphthalene and a small quantity of light oil and is afterwards discharged into the estuary.

Table I.

	(A) parts per 100	(B) parts per 100
Chloride as HCl	1·65	0·153
Alkalinity as CaO	0·014	0·20
Ammonia (free)	0·003	0·024
Ammonia (fixed)	0·002	0·003
Thiocyanate (as CNS)	nil	0·010
Thiosulphate (as S)	0·007	0·027
Cyanide (as HCN)	0·004	nil
Sulphur (as H_2S)	trace	trace
Monohydric phenols (as C_6H_5OH)	0·0105	0·184
Total tar-acids	0·0125	0·250
Oxygen consumed from $N/80$ permanganate at 80° F. in 4 hours	0·057	0·494
Oxygen consumed from $N/80$ permanganate at 80° F. in 3 minutes	0·020	0·315

The sulphur content of (A) may be as high in some samples as 0·01% H_2S.

(B) *Coke-oven effluent, semi-direct ammonia recovery.* In this type of plant the ammonia liquor which is deposited in the water-cooled condensers is separated from tar and is mixed with the condensate from the water-cooled condensers. The mixed liquor is distilled with steam in an ammonia still, with the addition of lime. The gases from the ammonia still are mixed with the crude coke-oven gas at a point in the gas main between the tar extractor and the saturator. The hot spent liquor from the ammonia still is discharged into the estuary.

Analyses, carried out by the Government Chemist, of samples of effluents

(A) and (B) are given in Table I (A) and (B). The total volume of effluents discharged per 24 hours is, very approximately, (A) 10,200 cubic metres and (B) 280 cubic metres.

(3) *Spent pickle liquor.* Pickle liquors are produced as the result of cleaning steel sheets or wire in concentrated hydrochloric acid, before galvanising. The following is an analysis of a spent pickle liquor (Table II).

Table II.

	Parts per 100
Total iron as Fe	16·05
Ferric iron	0·40
Ferrous iron	15·65
Free hydrochloric acid as HCl	3·13

In some works the free acid is neutralised with lime before discharge.

The volume of effluent produced varies widely according to trade conditions and, in addition, part of it is disposed of locally for iron recovery so that an average figure for the rate of discharge cannot be given.

The effect of polluting discharges.

After being discharged, polluting material is carried up and down the estuary under tidal action and it has been calculated that under normal conditions of freshwater flow from the upper river it may remain in the estuary for several days before finally reaching the open sea. During this time both sewage and industrial effluents undergo biological, or in the case of pickle liquors, chemical oxidation at the expense of the dissolved oxygen of the estuary waters. A belt of water, in the central portion of the estuary, moving to and fro under tidal action is thus permanently partially deoxygenated. The degree of deoxygenation has been found to depend in some measure on the freshwater flow and on certain other factors but mainly on the water temperature.

The possibility of migrating smolts being killed by dissolved oxygen deficiency was therefore one which had to be considered.

The lower limit of dissolved oxygen under which smolts can live for periods of several days does not seem to have been determined; such a determination would require the use of a large-scale deoxygenating apparatus and has not been attempted during the present investigation. It was found possible, however, to maintain a supply of running water having a dissolved oxygen content of 58 to 64 % of the saturation value (temperature 15·5°) and rainbow trout (*Salmo irideus* Gibb.), whose viability is of the same order as that of smolts, lived comfortably in this water for a period of 4 days. During the early part of the smolt migration of 1930, when the water temperature was low, smolts were found to be dying in water having a dissolved oxygen content of not less than 70 % of saturation. It appeared then that the mortality was due, at least in part, to some factor other than oxygen deficiency.

Investigations in progress at the Tees Survey Laboratory at Barnard

Castle and not yet published, have shown that fresh sewage or sewage undergoing aerobic decomposition when diluted with river water has no toxic action on fish for short periods.

Of the main types of trade wastes discharged it is improbable that pickle liquors contribute to the directly toxic substances in the estuary water except perhaps in restricted areas. The ferrous chloride they contain appears to be readily precipitated as insoluble compounds on admixture with the alkaline water of the estuary, since soluble iron salts have not been detected in the general body of the estuary waters. The p_H of the estuary water lies between that of the fresh water (about 7·0) and that of the sea (about 8·3) and is not appreciably affected by the free acids discharged.

A more detailed examination of the coke-oven effluents was therefore undertaken, with the object of determining the relative toxicity of the constituents of the two types.

The source of the toxicity of coke-oven effluents.

Rainbow trout, about 2″ to 4″ in length and 1 to 2 years old were used in all cases as the test animals. When placed in a solution containing a lethal concentration of a toxic substance they first display symptoms which are to some extent characteristic for the poison to which they are exposed. In all cases they eventually lose their equilibrium, after which they sometimes recover but finally remain in an overturned position. The length of time from their first exposure to the solution to this final overturning point is taken as a measure of the toxicity of the solution. All experiments were carried out in wide-mouthed vessels, a volume of either 12 or 20 litres of solution being used. In all cases the dissolved oxygen concentration was found, by analysis, to be near the saturation value and the hydrogen ion concentration was, if necessary, adjusted by the addition of acid or alkali, until the solution was approximately neutral.

The following experiments were carried out with a sample of direct process effluent having approximately the same composition as that given in Table I (A).

1. Toxicity of effluent: a 1 % solution caused trout to overturn in approximately 6 minutes.

2. The effluent was distilled in neutral solution: a 1 % solution of the distillate was approximately as toxic as the undistilled effluent.

3. The effluent was distilled in alkaline solution: a 1 % solution of the distillate was non-toxic for periods up to 60 minutes.

4. The residue from (3) was distilled in acid solution: a 1 % solution of the distillate was approximately as toxic as the undistilled effluent.

5. The effluent was distilled in acid solution: a 1 % solution of the distillate was approximately as toxic as the undistilled effluent.

6. The residue from (5) was distilled in alkaline solution: a 1 % solution of distillate was non-toxic for periods up to 60 minutes.

It appeared from these experiments that the toxic constituents of the effluent were volatile, and were capable of distillation from acid or neutral, but not from alkaline solution. Volatile neutral bodies and basic substances cannot have contributed appreciably to the toxicity of the effluent. An aqueous solution (A) was then prepared of the following composition:

Table III.

	g. per 100 cc.
Sodium chloride	2·73
Sodium thiosulphate	0·0345
Ammonium chloride	0·009
Phenol	0·0105
Mixed xylenols	0·002
Potassium cyanide	0·01

This mixture contains the main constituents of the waste liquor in the concentrations given in Table I (A). The toxicity of the artificial and natural effluents to trout was found to be approximately the same.

A series of solutions was then made up of the same composition as solution A (Table III), except that each constituent was in turn omitted, and the toxicities of these mixtures were determined. It was found that the toxicity was in each case practically the same as the complete solution A except when potassium cyanide was omitted, when a 1 % solution of the mixture was non-toxic under the conditions of the experiment. Moreover, a solution of pure potassium cyanide, in the concentration in which it occurs in the effluent, was found to have about the same toxicity as the complete effluent.

After the removal of cyanide by precipitation and filtration (as AgCN by the addition of $AgNO_3$) the liquor was non-toxic in a 1 % solution, the toxicity being restored to its original value by the addition of a quantity of potassium cyanide equivalent to the amount of cyanide removed. It appeared, then, that the cyanide fraction was the main toxic constituent of a typical direct process coke-oven waste liquor.

A similar investigation of the toxicity of the main groups of constituents of a typical semi-direct process coke-oven effluent was then carried out. The composition of the effluent was approximately the same as that given in Table I (B) and a 1 % solution of it caused trout to overturn in about 6 minutes. The effluent was distilled under the same conditions as those described for the direct process liquor and the toxicity to trout of the various distillates was determined. It was found that the poisonous constituents were volatile in acid or neutral, but not in alkaline solution. Double distillations showed that volatile neutral bodies did not appreciably contribute to the toxicity and it was concluded that the group of tar-acids was mainly responsible. It will be seen from Table I (B) that cyanide did not occur in the semi-direct process effluent examined, but that tar-acids were present in a very much larger concentration than in the direct process effluent.

It had now been shown that cyanides were the chief toxic constituents of

direct process, and tar-acids of semi-direct process, coke-oven effluents, and it was decided to determine the concentrations of these substances, together with the dissolved oxygen concentration, in the estuary waters where the mortality of smolts occurred during the 1931 migration.

In preparation for this work, some questions which appeared to have a bearing on the problem of smolt mortality were investigated and methods which might yield further information were examined; the main lines of investigation are summarised below.

The toxicity of potassium cyanide and p-*cresol.*

The toxicities to rainbow trout of various concentrations of potassium cyanide at temperatures of 5° and 10·5 to 12·5° and similar data for solutions of *p*-cresol (taken as a representative of the complex group of tar-acids) at 5° are shown in Fig. 1. In all these experiments 12 litres of solution were used and

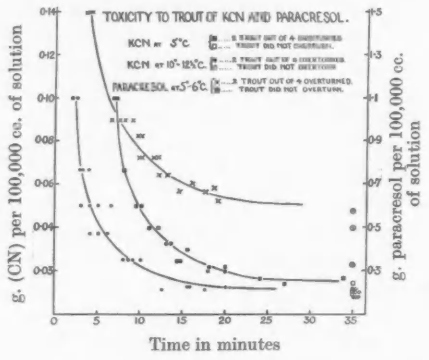
TOXICITY TO TROUT OF KCN AND PARACRESOL.
KCN at 5°C.
KCN at 10°-12·5°C.
PARACRESOL at 5°-6°C.
g. (CN) per 100,000 cc. of solution
g. paracresol per 100,000 cc. of solution
Time in minutes

Fig. 1.

the time taken by two fish out of four to overturn in it was observed. It will be noticed that, at the same temperature, *p*-cresol is very much less toxic than potassium cyanide; thus, trout overturned in less than 1 hour in a solution of potassium cyanide equivalent to 0·014 g. CN per 100,000 cc., but did not overturn in 1 hour in *p*-cresol at a strength of 0·5 g. per 100,000 cc.

It was found that given concentrations of potassium cyanide were more toxic at the higher temperature than at the lower. This relationship was more fully investigated over a wider range of temperature and it was found that the toxicity (represented by the reciprocal of the average time of overturning) was approximately proportional to the water temperature, over the temperature range 5 to 20°.

The toxicity of solutions of cyanide in water unsaturated with dissolved oxygen was also investigated and it was found that a solution of potassium cyanide became more toxic as the oxygen content was reduced.

The removal of cyanides from dilute solutions.

A method was worked out whereby cyanide in small concentration could be removed from the complex mixture of substances present in the waters of the estuary, and the effect of its removal on the toxicity of the water determined. Formaldehyde in sub-lethal concentration was added to combine with any cyanide present to form compounds which were non-toxic in the concentrations examined.

The effect of adding formaldehyde to the following substances was determined.

Potassium cyanide. Concentrations up to 1 g. CN per 100,000 cc. rendered non-toxic.

Phenol. Concentrations up to 1 g. phenol per 100,000 cc. No change in toxicity.

p-*cresol.* Concentrations up to 1·5 g. *p*-cresol per 100,000 cc. No change in toxicity.

Coke-oven effluents. 1 % solutions. No change in toxicity of semi-direct process effluent where the toxicity is due to tar-acids. Toxicity of direct process effluents, due to cyanide, destroyed.

Tees estuary water. Taken from the neighbourhood of industrial outfalls. Toxicity destroyed or materially reduced in cases where cyanides were found by analysis to be present.

It was decided, therefore, to examine samples of estuary water by this method during the 1931 smolt migration, in order to assess the importance of cyanides in contributing to their toxicity.

The examination of the colour of the gills of trout.

During the smolt migration of 1930, about 600 salmon and sea trout smolts, which had been picked up dead in the estuary or had been poisoned by known toxic substances in the laboratory, were examined and an attempt was made to determine the cause of their death from their *post mortem* appearance, but with no success, owing largely to the uncertainty as to how long the fish had been dead before examination. It was observed however that the gills of trout, when being poisoned in the laboratory by solutions of cyanides, or by effluents containing cyanides, appeared to be of a brighter colour than those of normal fish. Experiments were therefore carried out in which the gill colours of trout and sea trout smolts exposed to various known poisons were compared with a graded series of seven colours ranging from scarlet (Colour No. 2) to dark crimson (Colour No. 16). A definition of these colours on two colour scales is given in Note 1 at the end of this paper (p. 283). It was found that the gill colour became darker than that of normal fish when both rainbow trout and sea trout smolts were subjected to poisoning by tar-acids, naphthalene or dissolved oxygen deficiency but became markedly lighter when exposed to toxic con-

centrations of cyanides. It was decided therefore to match the gill colours of smolts found dying in the estuary during the migration of 1931 and to compare these with the gill colours of healthy smolts.

The 1931 smolt migration.

Smolt netting, carried out near the upper limit of salt water in the estuary, showed that smolts were migrating past that point from April 23rd to June 5th, 1931. In 1930 it was observed that the death of smolts occurred mainly, if not entirely, at high water in the reaches of the estuary between about 33,000 ft. and 73,000 ft. from the sea (*cf.* Fig. 2). In 1931 sampling was carried out daily

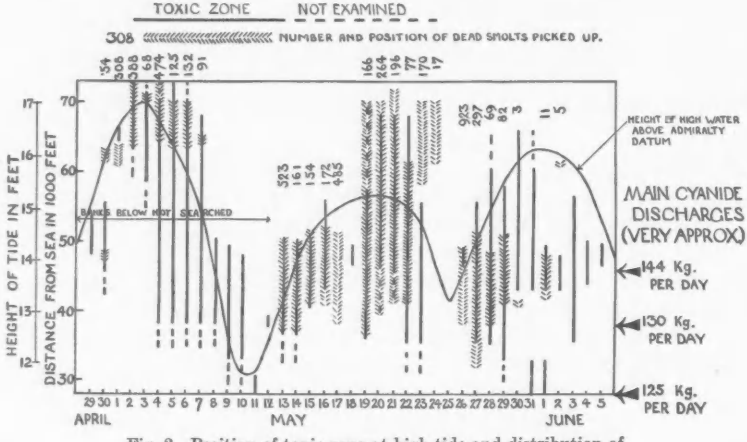

Fig. 2. Position of toxic zone at high tide and distribution of dead smolts at low tide in the Tees Estuary 1931.

in these reaches from April 25th to June 5th at about the time of high water, the following determinations being made.

1. Salinity: by titration with silver nitrate against standard sea water.
2. p_H value: colorimetrically.
3. Temperature.
4. Dissolved oxygen concentrations: by Winkler's method [1929] without preliminary oxidation.
5. Tar-acids (in most samples): by the method of Fox and Gauge [1920].
6. Cyanide: by phenolphthalin or silver nitrate methods (see note, p. 283).
7. Toxicity and toxicity due to cyanides: for this determination duplicate samples, each of 20 litres, were taken at each station. One sample was untreated: to the other 1·5 cc. of 40 % formaldehyde solution were added to remove cyanides, and the time taken by one rainbow trout to overturn in each solution was observed. The dissolved oxygen concentration was found to be approximately the same in the treated and untreated samples both at the beginning and the end of the toxicity determinations.

The results of the toxicity determinations are given in Table IV.

Table IV.

No. of samples Non-toxic: untreated Toxic: with formaldehyde	No. of samples Non-toxic: untreated Non-toxic: with formaldehyde	No. of samples Toxic: untreated Non-Toxic: with formaldehyde	No. of samples Toxic: untreated Toxic: with formaldehyde
5	140	130	8

Thus of 138 samples which were originally poisonous the toxicity of 130 was removed by formaldehyde. It will be noticed that 5 samples were innocuous untreated but were rendered toxic by the addition of formaldehyde. This is explained by the fact that the quantity of formaldehyde added (3 parts per 100,000) was only just below the minimum concentration toxic to trout, and incomplete mixing might result in a layer containing sufficient formaldehyde to be toxic. It is probable that some of the cases where toxicity was not removed by formaldehyde were also due to this factor. Other explanations can be advanced to account for the death of three fish in this group.

One case: the test fish used was a large sea trout smolt. It was removed in a helpless condition and subsequently died after having reduced the oxygen concentration to 13 % of the saturation value.

Two cases: both on the same day. The fish storage tanks had become foul and several fish died in them. The presumption is that the trout used for the experiment were in an unhealthy condition.

The experimental evidence therefore points to the conclusion that in practically all the samples examined, the toxicity, where present, was due to cyanides, this toxicity being destroyed by the addition of formaldehyde.

In Table V are shown the percentages of toxic samples found in various ranges of cyanide concentration.

Table V.

Concentration of cyanide; g. CN per 100,000 cc.	No. of samples	No. of toxic samples %	No. of non-toxic samples %
0	84	13	87
0·002	37	19	81
0·003–0·004	45	62	38
0·005–0·006	32	84	16
0·007–0·008	15	93	7
0·009–0·01	12	100	0
>0·01	23	100	0

The accuracy of the methods of determination of cyanides is discussed in a note at the end of this paper where it is shown that the concentration of cyanide found by analysis is, in general, lower than the amount actually present, especially at low concentrations of CN. The figures given in Table V are therefore only comparative.

The values of tar-acids found by analysis in the samples were generally of the order of 0·01 to 0·03 part per 100,000 the maximum observed value being

0·05. These concentrations are much lower than the minimum concentration of tar-acids toxic to trout (Fig. 1).

The dissolved oxygen concentration of the estuary waters during the period of migration was generally high for the time of year, owing to the abnormally low water temperature. The concentrations found in water in which smolts were actually observed to be dying are given in Table VI.

Table VI.

Dissolved oxygen concentration % saturation	Temperature range (° C.)	No. of days on which these concentrations were observed
> 70	10–14	2
60–70	10·5–13	3
50–60	11–12	7
40–50	11–13	3

It has been mentioned above (p. 275) that rainbow trout have been kept under laboratory conditions for 4 days in water having a dissolved oxygen concentration of 58 to 64 % of the saturation value. Other experiments have shown that trout are not killed in short periods in water containing only 20 % of the oxygen saturation value; similar results were obtained by Gardner [1926]. During the present investigations in 4 cases where smolts were used as test fish they were unharmed after 14 hours' exposure to formaldehyde-treated solutions in which they had reduced the oxygen content to 19–34 % of saturation. It is improbable therefore that the oxygen deficiency in the estuary was sufficient to have caused, by itself, the death of smolts.

Occasional samples of estuary water were aerated continuously during the period in which trout were exposed to them. In some cases the oxygen concentration rose nearly to the saturation value, but of these all samples which were found to contain cyanides remained toxic to trout.

During the migration, numbers of salmon and sea trout smolts were observed in an overturned condition or swimming feebly near the surface of the water: these were netted and their gill colours were compared with the colours of the standard chart (p. 283). Some of these smolts were transferred to freshwater tanks in the laboratory and the gill colours of the now healthy fish were recorded. The results are given in Table VII.

Table VII.

	Salmon smolts		Sea trout smolts	
	Dying	Normal	Dying	Normal
No. of smolts from which records were taken	66	40	131	176
Average gill colour	8·5	12·5	7·1	11·7

The brightening of the gill colour observed in the dying smolts was similar to that found in the laboratory in cases of cyanide poisoning.

From the whole of the data presented, it is concluded that cyanide is the main factor responsible for the smolt mortality in the estuary. Lehmann [1926], in his investigation on the Leine, has pointed out the hitherto underestimated importance of cyanides in the study of the pollution of rivers by industrial effluents.

The position of the toxic belt of the estuary during 1931 at about high water each day from April 29th to June 5th is shown in Fig. 2 together with the distribution of dead smolts on the banks at low water. The position of the toxic belt at high water appears to be determined mainly by the tidal range, partly by the volume of fresh water entering the estuary from the upper river and possibly by some other factors. The majority of smolts were killed at about the time of high water, and in dying they usually congregated near the banks, where they were left stranded as the tide ebbed. In general therefore they were found at low water in reaches which had been occupied by toxic water (the toxicity of which has been shown to be due to the presence of cyanide) at high tide.

The exceptions are probably explained by the fact that the position of the toxic zone was determined at only one high tide, occurring in daylight, of the two daily tides, and dead smolts were collected only during the daylight low water period. It is to be expected that roughly half the dead smolts found were killed during the unobserved high water period. Since the tidal range of the two daily tides is in general different, the position of the toxic belt and thus the distribution of dead smolts would be correspondingly altered.

Note 1. *The gill colour scale.*

The colours of the chart used for matching the gills of trout and smolts, referred to on p. 279, have been standardised by means of a Lovibond tintometer, modified for the examination of opaque objects (Table VIII). The nearest corresponding colours from Ridgeway's Colour Chart [1886] are also given for comparison.

Table VIII.

Gill colour scale colour No.	Lovibond tintometer units			Ridgeway's colours
	Red	Yellow	Blue	
2	15·8	1·5	0	Geranium red
4	15·3	1·5	0	
6	14·2	1·9	0	
8	12·8	1·9	0·3	
10	11·0	2·5	1·3	Burnt carmine
12	9·5	2·5	2·6	
14	8·9	2·8	3·4	
16	6·0	2·8	4·0	Indian purple

The comparison by the Lovibond tintometer was carried out by Mr Strafford of British Dyestuffs Corporation.

Note 2. *The determination of cyanides.*

The sample is distilled in acid solution and the concentration of CN in the distillate is determined by a modification of the phenolphthalin method due to Weehuizen [1905], an account of which will shortly be published. The reaction is not known to be given by any substance, other

than cyanides, likely to be present in the Tees Estuary, but it is inhibited by sulphides, which, if present, must be removed from the sample before distillation. If more than 0·01 g. CN per 100,000 cc. is found to be present, the estimation is checked by the silver nitrate method [Liebig, 1851]. The percentage difference between duplicate determinations by the two methods varies from 5 to 25 %, the larger discrepancies occurring in samples having a low CN concentration. A relatively large loss of CN occurs during the distillation of the sample which is common to both methods. Thus, a series of determinations of estuary water, originally cyanide-free, to which a known concentration of potassium cyanide, equivalent to 0·01 g. CN per 100,000 cc. had been added, gave results varying from 0·0035 to 0·006 g. CN per 100,000 cc. in different samples of estuary water.

The Prussian blue method is not sufficiently delicate for the estimation of the low concentrations of cyanides found in the estuary. Estimations of CN by the Prussian blue method have, however, been carried out on coke-oven effluents, and the values obtained compared with the concentrations determined by the phenolphthalin method in dilutions of these effluents in estuary water (Table IX).

Table IX. *g. CN per 100,000 cc.*

1	2	3	4	5
Concentration of CN in effluents by Prussian blue	Concentration of CN in diluting estuary water by phenol-phthalin	Concentration of CN in 0·25 % solution of effluent in estuary water by phenolphthalin	Observed concentration of CN in solution, due to added effluent (3)–(2)	Calculated concentration of CN in solution due to added effluent. From (1)
8·8	0·006	0·024	0·018	0·022
8·4	0·007	0·028	0·021	0·021
8·4	0·007	0·03	0·023	0·021
8·7	0·006	0·025	0·019	0·022

Summary.

1. The causes of the mortality amongst salmon and sea trout smolts migrating through the Tees estuary have been investigated.

2. The main directly toxic substances discharged into the estuary are tar-acids and cyanides from coke-oven effluents.

3. The smolt mortality has been shown to be due chiefly to cyanides.

Our thanks are due to the many local firms, authorities and individuals whose co-operation has made the work possible.

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XXXII. URICASE AND ITS ACTION.
V. FURTHER EXAMINATION OF OX-KIDNEY URICASE.

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Introduction.

It was shown in Part IV of this series [Truszkowski, 1930] that no uricolytic preparation could be separated from ox-kidney tissue by the action of aqueous solutions at p_H 6–11·5, by dissolution in alkali with subsequent reprecipitation by acid, or by elution with *n*-butyl alcohol; in all cases either the activity of kidney tissue was destroyed, or the extracts were inactive. It is, however, obvious that the conclusion that the whole of the insoluble constituents of kidney tissue are identical with uricase cannot be drawn on the basis of these experiments.

It is reasonably certain that all water-soluble kidney constituents are inactive, as was shown by experiments on centrifuged extracts. Both the fine particles in suspension and the exhaustively extracted residue of coarsely divided kidney tissue are active, the former much more so than the latter. The problem of the further purification and possible closer identification of uricase thus resolved itself into the problem of fractionating a mixture of insoluble substances.

There are three possible modes of attack of this problem—mechanical, chemical or biological. To the first belong methods such as sedimentation of suspensions or separation by dissection. Of the second type, the action of various factors which might dissolve inactive material, leaving a residue of more active uricase, or *vice versa*, will be described in this paper. The third method of attack would consist in administering to experimental animals poisons acting selectively on the glomeruli or tubules, and examining the kidneys in the usual way for uricase. It being impossible to use oxen as experimental animals, the success of this mode of attack depends on finding a laboratory animal possessing uricase in its kidneys, and of sufficient size to yield a workable amount of material. This limits the choice to dogs, cats or rabbits; as will be shown in the present paper, the kidneys of none of these animals contain appreciable quantities of uricase, so that, in the case of kidney-uricase, this method had to be abandoned. Work is, however, now in hand on the localisation of liver-uricase by an analogous method.

Experimental.

Uricase was prepared from ox-kidney by the methods described in Part IV of this series. Uric acid was determined colorimetrically by Folin's method. Solutions of uric acid were prepared by dissolving the acid in boiling saturated lithium carbonate, and making up to the desired volume with distilled water. The p_H of such solutions was 7·5–8·0.

(1) *Sedimentation of uricase suspensions.*

A litre of uricase suspension in chloroform water was left 24 hours in a cylinder at room temperature; 7 successive portions, every one of about 140 cc., were removed from the cylinder by means of a siphon and centrifuged, and 0·2 g. of the residue from every portion was shaken for 1 hour at 37° with 30 cc. of 0·05 % uric acid solution. The results, given in Table I, indicate that

Table I. *Sedimentation of uricase suspensions. Control systems contain 14·7 mg. uric acid.*

Successive portions from above down	Mg. uric acid		
	Found	Oxidised	Oxidised per g. uricase per hr.
1	6·98	7·72	38·6
2	7·35	7·35	36·7
3	6·98	7·72	39·6
4	6·6	8·1	40·5
5	7·5	7·2	36·0
6	6·98	7·72	38·6
7	8·62	6·08	30·4

only the lowest layer of suspension gives a comparatively less active preparation of uricase than the remaining six layers, the activities of the suspensions from which were very similar in all cases; since the lowest layer consisted chiefly of coarse particles, the relatively feebler action of this preparation might be due as much to the smaller surface of contact offered by unit weight of substance as to its higher content of inactive constituents. Further work in this direction was abandoned in view of the negative results obtained.

(2) *Preparation of uricase from isolated medullary and cortical tissue.*

On section of ox-kidney the medullary portions are visible as brownish-red patches, surrounded by greyish-yellow cortical tissue. The cortex was removed, practically free of medullary tissue, and the residue was as far as possible freed of cortical tissue. All fragments which appeared to contain both types of tissue were rejected, and the cortex and medulla were minced and shaken with chloroform water for 30 minutes; the suspensions were roughly filtered through wire gauze and centrifuged. The residue left by cortical suspensions was greyish-white in colour, whilst medullary uricase was brownish-yellow. 10 cc. of 1 % suspensions of both preparations were added to 20 cc. portions of uric acid, 2–3 cc. of toluene were added, and the flasks were left for 44 hours at 30°, after which

uric acid was determined. As is shown in Table II, cortical uricase is about 25 % more active than is medullary.

Table II. *Action of medullary and cortical tissues.*

Uricolytic preparation taken	Total volume of system cc.	Temp. and duration of exp.	Mg. uric acid: Initially present	Mg. uric acid: Found at end of reaction	Mg. uric acid: Oxidised
0·1 g. centrifuged medullary suspension residue	30	30° Stood 44 hrs.	24·6	4·35	20·25
0·1 g. centrifuged cortex	30	do.	24·6	0·42	24·18
2 g. washed extracted medullary tissue	25	34° Shaken 2 hrs.	33·1	11·7	21·4
2 g. washed extracted cortex	25	do.	33·1	3·6	27·5

The residues of coarsely divided kidney tissue (3–4 mm. cubes) left after filtering the suspensions were thoroughly washed in running water, and 2 g. of cortical and of medullary tissues were added to 25 cc. of uric acid, and the systems were shaken for 2 hours at 34°, after which uric acid was determined. As with kidney extract suspension residues, the activity of washed cortical tissue is about 25–30 % greater than is that of medullary tissue.

Whilst it is realised that it is not possible to effect complete separation of cortical from medullary tissue, or *vice versa*, by the rough methods employed, yet the results obtained are strong evidence that uricolytic properties are possessed by the cells of both parts of the kidney. The somewhat greater activity of cortical tissue may be due to its greater friability, as a result of which the dispersion of the catalyst is increased relatively to a greater extent during the reaction, as well as to the greater content of connective tissue elements in the medulla.

(3) *Action of ethyl alcohol and ether.*

Three 4 g. portions of uricase were left 24 hours at 37° with 30 cc. of alcohol, ether and water respectively, the suspensions were centrifuged, and 0·2 g.

Table III. *Action of C_2H_5OH and of $(C_2H_5)_2O$.*

Preparation taken	Mg. uric acid: Found	Mg. uric acid: Oxidised
Water-extracted uricase	3·37	5·33
Ether-extracted uricase	5·4	3·3
Alcohol-extracted uricase	6·6	2·1
Ether and alcohol extracts	8·7	0
Control	8·7	—

portions of the washed residues were shaken 90 minutes at 37° with 30 cc. of uric acid solution. The filtered ethereal and alcoholic centrifugates were evaporated at 18° under reduced pressure, and the colourless or faintly yellow oily residues were similarly shaken 90 minutes with 30 cc. of uric acid. The

results, given in Table III, indicate that the lipoidal constituents of kidney tissue are inactive, and that the activity of uricase is greatly reduced by contact with ether or alcohol.

(4) *Action of H·.*

It was shown in Part IV that uricase is soluble in alkaline solution, but that it is at the same time irreversibly inactivated. The possibility remained that either uricase or inactive constituents could be dissolved in acid solutions; before work could be done in this direction it was necessary to determine which concentrations of H· did not inactivate uricase. 10 cc. portions of McIlvaine's buffer solutions (p_H 2–7·6) were added to a number of centrifuge-tubes containing 10 cc. of 1 % uricase suspension, and the systems were centrifuged after keeping 1 hour at 37°, the centrifugates were taken for the determination of p_H (1·98, 2·67, 3·67, 4·28, 5·29, 6·56, 7·59), and the residues were suspended in 20 cc. portions of distilled water, and again centrifuged. 20 cc. portions of uric acid solution were added to the washed residues, as well as to an extra tube which had been previously wetted with water. Uric acid was determined in all systems after keeping for 15 hours at 37°, in the presence of toluene. 36·8 mg. were found in the control, and in all other systems except that treated with buffers at p_H 7·59, which contained 17·6 mg. (19·2 mg. oxidised). The p_H of all the final systems varied from 7·6 to 7·8.

The above result explains the initial rise in the p_H-oxidation curves, from p_H 6 to 7·4 [1930, Fig. 1, p. 1360]—the diminished oxidation of uric acid with diminishing p_H below 7·4 is due to increasing inactivation of the catalyst by H·; in the same way with p_H above 9·5 the rapid decline in activity is ascribable to inactivation by OH′.

A further conclusion to be drawn from the above experiment is that neither solutions more acid than p_H 6·56 nor more alkaline than p_H 9·5 can be used for the purification of uricase.

(5) *Action of oxidising agents, poisons, heavy metals,* etc.

Since the separation of uricase from inactive material cannot, as a result of its instability, be effected by the methods ordinarily applied to the separation of solid mixtures, the following experiments were performed, with the object of obtaining additional evidence as to its chemical nature. To 10 cc. portions of a 2 % suspension of cortical uricase in a number of 30 cc. centrifuge-tubes, 10 cc. of the following solutions (brought to p_H 7 by addition of *N* HCl or NaOH) were added: 0·5 % KCN, 0·01 *N* iodine, 0·1 *N* iodine, 3 % H_2O_2, 1 % $FeSO_4$, 1 % $CuSO_4$, 1 % $HgCl_2$, and water. The tubes were left 1 hour at 15°, centrifuged (10 minutes), the centrifugates were decanted off, and the residues were shaken three times with 15 cc. of distilled water, the suspension being centrifuged after each addition of water. 20 cc. of uric acid solution were added to every tube, as well as to a wet centrifuge-tube, taken as a control, and all tubes were left with toluene for 15 hours at 37°,

when uric acid was determined. The control system contained 24·0 mg. of uric acid, as did also the systems containing uricase previously treated with KCN, 0·01 and 0·1 *N* iodine, H_2O_2, $FeSO_4$ and $HgCl_2$. That containing uricase treated with $CuSO_4$ contained only 18·0 mg., whilst where water only had been added to uricase 16·4 mg. remained.

It follows that uricase is irreversibly inactivated by all the above reagents, with the exception of $CuSO_4$. In this case, two interpretations may apply—either uricase is not inactivated by $CuSO_4$, or it is inactivated, but the copper bound by protein or other material present enters into a new uricolytic complex. In view of the action of other heavy metals (Fe, Hg) the latter explanation appears to be more probable, and work on its experimental verification is now in hand.

(6) *Specificity of action of uricase.*

Since certain light might be thrown on the chemical nature of uricase by elucidating its action in oxidations other than that of uric acid, its action on oxalic acid and glycine was next studied. Should its action not be specific for uric acid, it might be possible to include it amongst the types of catalyst postulated by Quastel and Wooldridge [1927] or by Warburg [1928] in biological oxidations in general.

In the oxidation of glycine ammonia is evolved, and the rise in ammonia content of a system containing glycine and uricase would thus be an index of oxidation. 20 cc. of 5 % glycine solution were shaken for 5 hours at 37° with 10 cc. of 5 % uricase suspension, and the coloration given by the filtrate with Nessler's reagent was compared with that given by the filtrate of 20 cc. of glycine solution and 10 cc. of uricase suspension shaken separately, and mixed immediately before filtration. No difference was observed, indicating that under the given conditions no oxidation of glycine had taken place. In a second experiment air was aspirated for 48 hours at 37° through a number of pairs of cylinders, of which the first contained 10 cc. of 5 % uricase, 10 cc. of water, 20 cc. of borate buffer (p_H 9·5) and 25 cc. of 0·1 *N* H_2SO_4 respectively, the second the same, substituting 10 cc. of alkaline 5 % glycine for uricase, and acid, and the third glycine and uricase together. The acid in the second cylinder of every pair was then titrated, 11·2 cc. of 0·1 *N* acid being found to have been neutralised in the first and third cylinders, and none in the second. These results indicate that an alkaline gas is evolved by uricase in alkaline solution, and that uricase has no oxidative or deaminative action on glycine.

In the case of oxalic acid, three systems containing respectively 10 cc. each of 2 % uricase suspension and of 0·5 % ammonium oxalate, 10 cc. of uricase and of water, and 10 cc. of oxalate and of water were left for 48 hours at 27°, and filtered, and the number of cc. of 0·05 *N* $KMnO_4$ required to oxidise 10 cc. of the filtrates was determined. 16·9 cc. of $KMnO_4$ were used in the case of oxalate alone, 1·0 cc. in that of uricase alone, and 17·8 cc. where both were present together. It follows that the oxidation of oxalic acid is not catalysed by uricase.

(7) *Temperature of inactivation of uricase.*

1 % suspensions of cortical uricase were kept for 5 minutes in water at 20, 30, 40, 50, 60, 70, 80, 90 and 100°, and 20 cc. of uric acid solution were added to the cooled suspensions, which were then kept with toluene for 15 hours at 37°. The control system contained 20·7 mg. of uric acid, as did also those containing uricase heated at 80–100°, whilst 0·15 mg. was oxidised at 70°, 1·35 mg. at 60° and 14·4 mg. at 20–50°, indicating that the inactivation temperature lies between 50 and 60°. This result is in agreement with Batelli and Stern's [1909] findings for ox-kidney uricase.

(8) *Action of KCN and of $Na_4P_2O_7$.*

It has been shown in Part III of this series that the action of Fe-containing active charcoal on uric acid is largely inhibited by KCN but not by $Na_4P_2O_7$. Warburg and Sakuma [1923] and Meyerhof and Matsuoka [1924] have, however, shown that the oxidation of cysteine and of laevulose by traces of metal is inhibited by KCN and $Na_4P_2O_7$ when Fe is present, but only by KCN when Cu is present. Whilst, therefore, the differentiation of these metals by the use of KCN and $Na_4P_2O_7$ is not universally applicable, yet it is of interest to examine the effects of these reagents on uricase.

With this object, a number of systems were set up, containing 10 cc. of uric acid, 10 cc. of 2 % uricase suspension, and 10 cc. of *M*/166 KCN, 0·21 *M* $Na_4P_2O_7$, or water (all at p_H 7·5), and uric acid was determined after shaking for 90 minutes at 30°. 23·8 mg. were found in the control systems, containing 10 cc. of uric acid solution and 20 cc. of water, 23·7 mg. in the presence of KCN, and 17·25 mg. in the systems containing $Na_4P_2O_7$ or water.

(9) *Uricolytic action of kidneys of dog, rabbit and cat.*

The uric acid content of systems containing 20 cc. of uric acid and 0·1–1 g. of the residues from kidney extract centrifugates (medulla and cortex taken separately) was determined before and after shaking for 3–5 hours at 40°; in no case was any difference observed. Uricase is not therefore present in appreciable quantity in the kidneys of these animals.

Discussion.

The above experiments demonstrate the impossibility of the purification or concentration of ox-kidney uricase by any of the methods commonly used; the unusual sensitiveness of uricase to $H^{\cdot}$ and OH', oxidising agents, heavy metals, *etc.*, renders any but the mildest chemical methods impracticable. At the same time, sufficient material is available to allow of certain conclusions as to the chemical nature of uricase.

Both the extreme instability of uricase and the results obtained with ether and alcohol extracts make it improbable that the active substance is a lipoid.

The insolubility of the active factor excludes a whole series of extractive substances, leaving only proteins to be considered.

Uricase is, therefore, in all probability a protein of highly specific and delicate surface configuration. The inhibitory action of KCN points to the presence of a heavy metal in this protein, and the fact that sodium pyrophosphate does not inhibit reaction suggests that this metal may be copper. The inactivation temperature (50–60°) is one at which many other enzymes and proteins undergo denaturation.

As to the site of action of uricase in the kidney, little definite information is available. It is present in both the cortex and the medulla, that derived from the former being more active, whence it might be concluded that it is a constituent of the epithelium of the renal tubules.

Summary.

1. Ox-kidney uricase suspensions do not yield fractions of different activity on sedimentation.

2. Both medullary and cortical tissues are active, the action of the latter being, however, about 25 % greater than that of the former.

3. Alcohol and ether extracts of uricase are inactive; the residues are less active than is the unextracted material.

4. Uricase is irreversibly inactivated by contact with solutions of p_H 2–6·56, but not of p_H 7·59.

5. Uricase is irreversibly inactivated by neutral solutions of KCN, iodine (0·01 and 0·1 *N*), H_2O_2, $FeSO_4$ and $HgCl_2$, but not by $CuSO_4$.

6. Uricase has no action on acid and alkaline glycine solutions, or on alkaline oxalate solutions.

7. Uricase is completely inactivated by heating for 5 minutes at temperatures above 70°, almost completely inactivated at 70°, partly at 60°, and not at all at 50° or less.

8. KCN completely inhibits uricolysis by ox-kidney-uricase, whilst $Na_4P_2O_7$ has no effect.

9. Dog, rabbit and cat kidneys do not contain uricase.

The author has much pleasure in acknowledging his gratitude to Prof. St J. Przyłęcki for his constant interest in the prosecution of this research.

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XXXIII. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.

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Of the several colorimetric methods for the determination of phosphorus which have been described during recent years, probably the Briggs [1922] modification of the Bell and Doisy [1920] method and the method of Fiske and Subbarow [1925] have met with most general favour. Both these methods depend on the reduction of phosphomolybdic acid to give a blue colour, the intensity of which is proportional to the concentration of phosphate.

Martland and Robison [1926] proposed a very convenient modification of the Briggs procedure, in which the colour was allowed to develop at an acidity higher than that usually used. Although the total amount of colour was not as great as that developed at lower acidities, a much greater variation in acidity was allowable without any appreciable variation in the amount of colour produced. Consequently, there was no necessity of adjusting the acidity of the test solution, or of adding trichloroacetic acid to the standard to make it similar to the test in acidity and composition. By keeping the sulphuric acid and ammonium molybdate separate they were able to use the same solutions for "free" and for "total" phosphorus. In the latter case the sample was digested in a boiling-tube with the amount of sulphuric acid (1 cc. of 30 %) used in bringing up the colour, and the oxidation completed by adding a drop of 30 % hydrogen peroxide. The small amount of sulphuric acid lost during the digestion was not sufficient to cause any disproportionality in the colour produced, so that it was only necessary to wash the contents of the tube into a 15 cc. volumetric flask and add the 1 cc. of 10 % ammonium molybdate and 1 cc. of 0·5 % quinol in 20 % sodium sulphite to bring up the blue colour, which was usually read after 20 or 30 minutes.

Fiske and Subbarow pointed out several objections to the use of quinol as the reducing agent, such as the necessity of allowing 30 minutes for the colour to develop, and the lack of proportionality of colour produced with solutions of different strength in the Briggs procedure. They proposed the use of 1:2:4-aminonaphtholsulphonic acid, which has the great advantages of giving the full complement of blue colour in 5 minutes or less, and of giving excellent proportionality of colour over a wide range of concentration of phosphate ion. It has the added advantage, moreover, of being far less affected by various

substances, *e.g.* ammonium and iron salts, nitrites and nitrates, chlorides and silicates, all of which interfere in varying ways with the colour production when quinol is used as the reducing agent.

The method in use in this laboratory is a combination of the Martland and Robison and the Fiske and Subbarow procedures and is believed to have advantages over the methods previously described. The high acidity recommended by Martland and Robison has been retained, and the acid and molybdate are kept separate. The reducing agent used is the aminonaphtholsulphonic acid suggested by Fiske and Subbarow, but is made up in a slightly different way, since it has been found impossible to keep the sulphonic acid in solution in the proportions of bisulphite and sulphite recommended.

It is in the estimation of total phosphorus, however, that the chief variation in method has been introduced. Various workers have recommended the destruction of the organic matter by sulphuric and nitric acids, sulphuric acid and hydrogen peroxide, nitric acid and nitrates, by alkaline fusion mixtures and by dry incineration. For most biochemical work the sulphuric acid and hydrogen peroxide method is probably the best. Recently, however, there have come on the market concentrated preparations of perchloric acid ($HClO_4$) which are now readily obtainable in very pure form and of a strength suitable for destruction of organic material. Perchloric acid has definite advantages as an oxidising agent which are not possessed by other acids. Its 60 % solution is a heavy, colourless liquid, much of the appearance of concentrated sulphuric acid, though not so viscous as not to be easily used with a pipette. It distills at something over 200°, and is a much better oxidising agent at high temperature than is sulphuric acid, having four available oxygen atoms, while sulphuric acid has only one. At room temperatures it is practically inert, showing no tendency to oxidise materials unless strongly heated. Its formula weight is nearly the same as that of sulphuric acid, but since it is univalent, while sulphuric is bivalent, twice the amount of perchloric acid is used to give the same acidity. This extra bulk of acid has a definite advantage in the oxidation of organic matter in a boiling-tube, since there is less danger of superheating both in the liquid and at the glass surface just above the acid layer; bumping and spattering are consequently much less apt to occur than when sulphuric acid is used, and the etching of a ring near the bottom of the test-tube, which may be quite marked in tubes which have been used for a long time with sulphuric acid, is very seldom found with perchloric acid. The oxidation is not only smoother, but is much more rapid and complete and the use of any hydrogen peroxide is usually quite unnecessary.

Another considerable advantage in the use of perchloric acid, both for free and total phosphorus determinations, is that most of the salts of this acid are very soluble. The isolation of biological materials as their barium salts is much used in biochemical work, and the barium must, of course, be removed before an analysis can be made in which sulphuric acid is to be used. Barium perchlorate, however, is very soluble in water and by substituting perchloric for

sulphuric acid in the estimation of both the free and total phosphorus of barium salts, the necessity for removing the barium is eliminated.

Method.

Solutions required. 72 % or 60 % perchloric acid (the strength commonly obtainable on the market to-day). 1 cc. of 72 % or 1·2 cc. of 60 % perchloric acid contain almost the same "total acidity" as 1 cc. of 30 % (by volume) sulphuric acid (the amount used by Martland and Robison).

5 % ammonium molybdate.

0·2 % aminonaphtholsulphonic acid. 0·5 g. of the 1:2:4-acid, 30 g. sodium bisulphite and 6 g. crystalline sodium sulphite are dissolved by shaking with enough water to make 250 cc. If the solution does not filter clear it should be left overnight and again filtered. A fresh solution should be prepared every 2 weeks.

Standard phosphate. A stock solution is made by dissolving 2·1935 g. of pure potassium dihydrogen phosphate in 500 cc. of water. This solution contains 1·0 mg. P per cc. A dilute standard solution is made by diluting 5 cc. of the stock solution to 500 cc. with water. 10 cc. of this solution contain 0·1 mg. P. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might otherwise cause a loss of inorganic phosphate.

Procedure.

Inorganic phosphate. An amount of the solution to be tested is measured into a 15 cc. volumetric flask and water added to about 10 cc. 1 cc. of 72 % or 1·2 cc. of 60 % perchloric acid, 1 cc. of molybdate and 0·5 cc. of sulphonic acid are added, and water to 15 cc. A standard containing an appropriate amount of phosphate (5 or 10 cc. of the dilute standard solution equivalent to 0·05 or 0·10 mg. P) is prepared at the same time and in the same way. The contents of the flasks are gently shaken between each addition, and finally mixed by inverting and shaking. The colours are read after 5 minutes in a Duboscq colorimeter.

For the determination of inorganic phosphate in trichloroacetic acid filtrates of blood[1], urine and other solutions where there is no barium present, it may be preferable to use sulphuric acid instead of perchloric acid. In this case it is convenient to use a mixed solution of 5 % ammonium molybdate in 15 % (by volume) sulphuric acid. 5 or 10 cc. of protein-free blood-filtrate, 0·2 or 0·5 cc. of urine are treated with 2 cc. of the molybdate-sulphuric acid mixture[2], 0·5 cc. of sulphonic acid and water to 15 cc.

[1] Usually prepared by shaking 1 volume of oxalated blood with 4 volumes of 10 % trichloroacetic acid and filtering. 5 cc. of filtrate represent 1 cc. of blood.

[2] It has been found advisable to employ this larger amount of molybdate, in the presence of sulphuric acid, since the colour is much slower to develop with sulphuric acid than with perchloric acid when only 1 cc. of 5 % ammonium molybdate is used. With 2 cc. of 5 % molybdate, however, the colour develops at almost the same rate in the presence of either sulphuric or perchloric acid. The smaller concentration of molybdate is used with perchloric acid because the permissible variation in acidity is greater, while the total amount of colour produced is almost the same.

Table I. *Colorimetric readings by perchloric acid method in presence of interfering substances.*

Standard 0·1 mg. P, 1·2 cc. 60 % $HClO_4$, 1 cc. 5 % ammonium molybdate, and 0·5 cc. sulphonic acid in a volume of 15 cc. Standard set at 20 mm.

Time (min.)	$HClO_4$ −0·4 cc.	$HClO_4$ −0·2 cc.	$HClO_4$ Standard amount	$HClO_4$ +0·2 cc.	Substance added: 25 % $CCl_3.CO_2H$ 1 cc.	25 % $CCl_3.CO_2H$ 2 cc.	25 % $CCl_3.CO_2H$ 3 cc.	*M* NaCl	*M*/3 $NaNO_3$	*M*/3 $(NH_4)_2SO_4$	Na_2SiO_3 5 mg. SiO_2
					0·04 mg. P (theoretical reading 50 mm.)						
2	—	50·1	50·0	—	50·0	49·9	—	—	—	—	47·0
5	47·5	50·1	49·2	50·2	50·1	50·1	—	—	—	—	46·6
10	47·1	50·9	49·2	—	50·1	50·0	—	—	—	—	46·6
15	47·3	51·3	—	50·4	49·9	49·8	—	—	—	—	—
20	47·2	51·1	49·2	50·3	50·1	49·8	—	—	—	—	—
30	46·5	50·6	—	50·2	50·1	49·9	—	—	—	—	—
					0·08 mg. P (theoretical reading 25 mm.) +50 mg. per 100 cc. $K_2C_2O_4$						
2	25·6	—	25·0	—	—	25·1	—	24·5	24·7	24·6	—
5	—	—	25·0	—	—	24·9	—	24·6	24·6	24·5	24·0
10	24·9	—	25·1	—	—	24·6	—	—	24·7	24·5	23·5
15	25·1	—	25·4	—	—	—	—	24·1	24·9	—	—
20	25·1	—	25·4	—	—	—	—	—	25·1	—	23·4
30	25·4	—	—	—	—	24·4	—	24·1	25·0	24·0	—
					0·12 mg. P (theoretical reading 16·7 mm.)						
2	—	16·5	16·9	16·8	16·5	—	—	—	—	—	—
5	16·9	16·7	16·8	16·7	16·7	16·9	16·6	—	—	—	—
10	17·0	16·6	16·9	16·6	16·5	16·5	16·3	—	—	—	—
15	17·0	16·5	17·0	16·6	16·5	16·5	16·4	—	—	—	—
20	17·1	16·5	—	16·5	16·6	16·5	16·4	—	—	—	—
30	17·1	16·6	17·0	16·6	16·5	16·6	16·4	—	—	—	—
					0·25 mg. P (theoretical reading 8·0 mm.)						
2	—	8·1	—	—	8·1	—	—	—	—	—	—
5	8·3	8·1	8·1	8·2	8·1	8·4	8·5	—	—	—	—
10	8·2	8·0	8·1	8·1	7·9	8·2	8·2	—	—	—	—
15	8·3	8·1	8·1	8·1	7·9	8·1	8·1	—	—	—	—
20	8·2	8·0	8·1	8·1	7·9	8·1	8·1	—	—	—	—
30	8·2	8·0	8·1	8·1	8·0	8·0	8·1	—	—	—	—

Total phosphate. The sample is measured into a boiling-tube of about 1 × 6 in. and of good acid-resistant glass, such as monax or pyrex. 1·2 cc. of 60 % perchloric acid are added and the contents of the tube heated with a micro-burner or on an electric heater[1]. The contents of the tube become concentrated, turn brown and then, as the temperature rises and the acid begins to fume they become colourless, the organic matter being completely oxidised in a few minutes. In some cases where the amount of organic material is large and the oxidation slow it may be necessary to add a drop or two of nitric acid or of 30 % hydrogen peroxide; in this case it will be necessary to continue the heating for 3 or 4 minutes after the mixture has become colourless, in order to drive off the excess of nitric acid or hydrogen peroxide. The cooled contents of the tube are washed with several portions of water into a 15 cc. volumetric flask.

It has been found that about 0·2 cc. of the perchloric acid is lost during the course of heating in an average determination. While the loss of this amount of perchloric acid will not appreciably affect the colour produced, it is thought advisable to add this amount of extra acid in order to attain approximately the same concentration as that in the standard, and to insure against the possibility of overstepping the safe limits of variation in acidity.

1 cc. of molybdate and 0·5 cc. of sulphonic acid are added to the test and at the same time a standard is prepared from an appropriate number of cc. of the dilute standard solution, 1·2 cc. of perchloric acid, 1 cc. of molybdate and 0·5 cc. of sulphonic acid. Test and standard are diluted to the mark, mixed, and read after 5 minutes.

Some results with the method, using perchloric acid in the presence of several interfering substances, are given in Table I. The non-interference of

Table II. *Results of analyses of barium salts of phosphoric esters by perchloric acid method.*

Substance	P present (calc.) mg.	P found mg.	P theoretical %	P found %
Glycerophosphate	0·1010	0·1000 0·0996	10·1	10·0 9·96
Glycerophosphate, H_2O	—	—	9·55	9·45 9·62
Ethyl phosphate	—	—	11·88	11·87
Ethyl phosphate, H_2O	0·2903	0·2914 0·2941	11·11	11·13 11·24
Ethyl phosphate, H_2O	0·1443	0·1439 0·1419	11·11	11·08 10·93
Phenyl phosphate, $2H_2O$	0·0942	0·0938 0·0940	8·98	8·95 8·97
Phenyl phosphate, $2H_2O$	0·0931	0·0930 0·0932	8·98	8·97 8·99

[1] A very convenient electric heater has been described by Stanford and Wheatley [1925]. The elements for this heater can be obtained from Messrs Leonard Toomer and Co., Corporation Chambers, 54 Lower Thames Street, London, E.C. 3.

various substances with the colour produced with 1:2:4-aminonaphtholsulphonic acid when sulphuric acid is used has been amply demonstrated by Fiske and Subbarow and by Vasarhelyi [1929]. Table II contains the results of total phosphorus determinations on the barium salts of several phosphoric acid esters in which the organic material was destroyed by digesting with perchloric acid.

SUMMARY.

A procedure is proposed for the colorimetric determination of phosphorus in which sulphuric acid is replaced by perchloric acid, which is a much better oxidising agent for the destruction of the organic material in total phosphorus determinations. The colour is developed at a high acidity which allows of considerable variation without any loss in the proportionality of colour produced; hence no allowance is necessary for the presence of moderate amounts of trichloroacetic acid in test solutions. The full complement of blue colour is brought up in about 5 minutes by the use of 1:2:4-aminonaphtholsulphonic acid as the reducing agent.

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XXXIV. THE BODY FATS OF THE PIG.

II. SOME ASPECTS OF THE FORMATION OF ANIMAL DEPÔT FATS SUGGESTED BY THE COMPOSITION OF THEIR GLYCERIDES AND FATTY ACIDS.

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In order to obtain evidence of the glyceride structure of pig depôt fats parallel with that for tallows [Banks and Hilditch, 1931] it was necessary, owing to the relatively small proportions of fully-saturated glycerides, to employ much larger quantities of material than were available in the group of pig depôt fats, the component fatty acids of which were recently described [Bhattacharya and Hilditch, 1931]. The whole of the leaf fat and a complete longitudinal section of the back fat, about 20 cm. wide, from a sow reared at the National Institute for Research in Dairying were therefore procured in order to make a detailed survey of the component glycerides in the perinephric fat and in the layers on either side of the central seam of connective tissue in the back fat. The diet of the animal for about 15 months prior to slaughter had consisted of a mixture of wheat middlings (6–12 parts), barley meal (2½–5 parts) and fish-meal (½–1 part) with about 1 part of either extracted soya bean meal, dried milk, or cheese.

The fatty tissue was extracted twice by boiling with acetone, after which the residual tissue was thoroughly pressed and finally washed three times on a filter with hot acetone: the last traces of acetone were subsequently removed by heating under vacuum at 100°.

Table I.

	Extraction			Extracted fat			Mixed acids
	Tissue g.	Fat obtained g.	Yield %	Saponification equivalent	Iodine value	M.P. (open tube) °C.	Setting-point °C.
Outer back:							
shoulder	575	359	60	287·1	76·9	31·5	33·5
main central	1915	1259	66	287·1	72·6	31·9	36·0
tail	420	247	60	287·3	72·0	33·8	36·8
Inner back:							
shoulder	490	452	90	286·5	71·1	35·0	38·6
main central	2580	2373	92	284·7	64·6	39·0	40·9
tail	375	346	92	283·1	64·6	37·0	40·8
Leaf	2400	2333	97	284·1	59·0	48·0	43·6

The shoulder end of the back fat was noticeably softer than the main central portion and therefore about one-sixth of each end of both layers was extracted and examined for component fatty acids separately from the remainder. The fats isolated from the various parts of the animal are described in Table I.

Component fatty acids of the sow depôt fats.

The method of determination was the same as that employed in the previous work on tallows and pig fats [Banks and Hilditch, 1931; Bhattacharya and Hilditch, 1931], and was described in detail in the former communication (p. 1170). A summary of the final analytical data for the component fatty acids of each of the above pig fats is given in Table III.

It will be observed that in each instance small proportions of highly unsaturated acids of the C_{20} and C_{22} series are included. The presence of these derivatives became evident during the fractional distillation of the methyl esters of each set of "liquid" (mainly unsaturated) acids (obtained in the preliminary lead salt separation), the iodine values and equivalents of the final fractions and residual esters being greater than the corresponding values for the C_{18} unsaturated esters present. To illustrate this, and also the manner in which the amount of C_{20-22} esters was estimated, we give in Table II the fractionation data for the "liquid" esters of (i) the beef tallow "N" quoted in our earlier paper (p. 1170), (ii) the fat from the inner back layer of pigs fed on a diet containing 3 % of arachis oil [Bhattacharya and Hilditch, 1931], and (iii) the fat from the main central portion of the inner back layer of the sow dealt with in this paper.

Table II.

	From beef tallow "N"			From inner back layer arachis oil diet			From inner back layer. Present series		
Fraction no.	g.	Saponification equivalent	Iodine value	g.	Saponification equivalent	Iodine value	g.	Saponification equivalent	Iodine value
1	36·94	273·8	69·4	3·05	276·6	83·2	32·83	284·8	89·6
2	11·63	285·5	82·5	7·56	289·5	97·4	10·36	292·0	102·1
3	14·11	291·7	85·0	9·11	293·8	101·4	10·40	292·8	103·3
4	9·11	292·9	86·3	7·59	295·0	102·7	10·39	292·6	105·2
5	9·55	292·6	86·3	11·54	295·2	105·5	10·48	293·1	106·0
6	10·46	293·8	88·2	7·00	302·4	107·4	9·82	294·6	106·6
7	8·88	294·0	89·0		(296·0)	(106·9)	7·80	297·9	109·0
8	8·58	296·3	90·2				7·45	317·2	128·1
9	8·65	320·6	88·6					(305·6)	(125·2)

The figures in brackets are the corresponding observed values for the residual esters after removal of unsaponifiable material, and show that esters of higher equivalent and iodine value occur in the final fractions of the "liquid" esters of the present series. The fatty acids from the latter were also treated in ether solution with bromine, when crystalline bromine addition compounds were precipitated: these were almost insoluble in boiling benzene, and when heated, blackened, but did not melt, at 225–230°. This is characteristic of the

polybromides of the highly unsaturated marine animal oil acids of the C_{20} and C_{22} series. The proportion of these present was therefore estimated approximately from the mean iodine values of the C_{18} and C_{20-22} groups of esters present in each case. (The ester fraction of equivalent nearest to, but below, 296 was assumed to be composed only of C_{18} unsaturated esters; the mean iodine value of the C_{20-22} esters was deduced from the residual esters (freed from unsaponifiable matter) on the assumption that their mean equivalent [compare Guha, Hilditch and Lovern, 1930] was approximately 330.) The latter assumption, although of an approximate order, introduces little error, since the amount of C_{20-22} esters present is in all cases relatively very small; the calculated iodine values of the C_{20-22} esters present ranged from 170 to 240.

Table III. *Summarised data for component fatty acids of the pig fats.*

Acid	Solid acids *S*	Liquid acids *L*	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mols.)
	Outer back fat, shoulder end (200·0 g.)*.				
	(25·0 %)	(75·0 %)			
Myristic	0·12	4·32	4·44	4·4	5·3
Palmitic	18·50	—	18·50	18·5	19·8
Stearic	5·40	—	5·40	5·5	5·3
Oleic	0·98	53·16	54·14	54·2	52·8
Linoleic	—	15·25	15·25	15·3	15·0
C_{20-22} unsaturated	—	2·14	2·14	2·1	1·8
Unsaponifiable	—	0·13	0·13	—	—
	Outer back fat, main central portion (199·0 g.).				
	(29·4 %)	(70·6 %)			
Myristic	0·25	3·53	3·78	3·8	4·6
Palmitic	20·02	0·28	20·30	20·3	21·7
Stearic	7·92	—	7·92	7·9	7·6
Oleic	1·21	52·81	54·02	54·1	52·6
Linoleic	—	12·99	12·99	13·0	12·7
C_{20-22} unsaturated	—	0·92	0·92	0·9	0·8
Unsaponifiable	—	0·07	0·07	—	—
	Outer back fat, tail end (194·5 g.).				
	(31·1 %)	(68·9 %)			
Myristic	0·59	3·72	4·31	4·3	5·2
Palmitic	22·15	—	22·15	22·2	23·7
Stearic	7·23	—	7·23	7·3	7·0
Oleic	1·13	47·96	49·09	49·2	47·7
Linoleic	—	15·26	15·26	15·3	14·9
C_{20-22} unsaturated	—	1·73	1·73	1·7	1·5
Unsaponifiable	—	0·23	0·23	—	—
	Inner back fat, shoulder end (204·0 g.).				
	(32·3 %)	(67·7 %)			
Myristic	0·15	4·00	4·15	4·2	5·0
Palmitic	22·74	—	22·74	22·8	24·3
Stearic	8·59	—	8·59	8·6	8·3
Oleic	0·82	46·64	47·46	47·5	46·0
Linoleic	—	15·59	15·59	15·6	15·3
C_{20-22} unsaturated	—	1·32	1·32	1·3	1·1
Unsaponifiable	—	0·15	0·15	—	—

Table III (*cont.*).

Acid	Solid acids *S*	Liquid acids *L*	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mols.)
	Inner back fat, main central portion (203·0 g.).				
	(37·7 %)	(62·3 %)			
Myristic	0·33	3·48	3·81	3·8	4·6
Palmitic	25·48	0·45	25·93	26·0	27·7
Stearic	11·01	—	11·01	11·0	10·6
Oleic	0·88	43·11	43·99	44·1	42·6
Linoleic	—	13·55	13·55	13·6	13·2
C_{20-22} unsaturated	—	1·53	1·53	1·5	1·3
Unsaponifiable	—	0·18	0·18	—	—
	Inner back fat, tail end (201·0 g.).				
	(37·8 %)	(62·2 %)			
Myristic	0·09	4·23	4·32	4·3	5·2
Palmitic	23·25	—	23·25	23·3	24·9
Stearic	13·78	—	13·78	13·8	13·3
Oleic	0·68	42·77	43·45	43·5	42·1
Linoleic	—	13·87	13·87	13·9	13·5
C_{20-22} unsaturated	—	1·20	1·20	1·2	1·0
Unsaponifiable	—	0·13	0·13	—	—
	Leaf (perinephric) fat (202·0 g.).				
	(45·8 %)	(54·2 %)			
Myristic	—	3·96	3·96	3·9	4·7
Palmitic	27·66	—	27·66	27·7	29·4
Stearic	17·56	—	17·56	17·6	16·9
Oleic	0·58	35·06	35·64	35·7	34·5
Linoleic	—	13·66	13·66	13·7	13·3
C_{20-22} unsaturated	—	1·41	1·41	1·4	1·2
Unsaponifiable	—	0·11	0·11	—	—

* Weight of mixed fatty acids employed in the analysis.

The different component acids of each of the present group of fats exhibit numerical relationships of an exactly similar kind to those discussed in the former part of this series [Bhattacharya and Hilditch, 1931, Table III, p. 1954], as will be seen from Table IV.

The most notable feature is, perhaps, the tendency for the united molar percentage of stearic, oleic and linoleic acids to be in the neighbourhood of 70 %. Where the total molar content of saturated acids is highest (51 %, perinephric fat), that of the united C_{18} acids is lowest, and conversely: but whereas the most unsaturated (outer back, shoulder) fat contains little more than half as much saturated acids (30 %) as the perinephric fat, its molar content of C_{18} acids has only increased to 73 %. The increase, between these two extreme cases, of about 8 % in an otherwise tolerably constant proportion of C_{18} acids is mainly compensated for by some diminution in the amount of palmitic acid, the myristic acid figures remaining throughout at about 5 %.

It should next be observed that, as in the previous instances, the increase in stearic acid is mainly at the expense of oleic, and not linoleic, acid, the amount of the latter increasing only slightly (from 13 to 15 %) with increasing unsaturation of the fats. This is additional evidence that the comparatively

Table IV.

(i) Molar distribution of individual acids.

Fat	Myristic	Palmitic	Stearic	Oleic	Linoleic	C_{20-22} unsaturated
Outer back						
shoulder	5·3	19·8	5·3	52·8	15·0	1·8
central	4·6	21·7	7·6	52·6	12·7	0·8
tail	5·2	23·7	7·0	47·7	14·9	1·5
Inner back						
shoulder	5·0	24·3	8·3	46·0	15·3	1·1
central	4·6	27·7	10·6	42·6	13·2	1·3
tail	5·2	24·9	13·3	42·1	13·5	1·0
Leaf (perinephric)	4·7	29·4	16·9	34·5	13·3	1·2

(ii) Molar percentages of the various groups of acids.

	Total saturated acids	Total C_{18} acids	Stearic acid	% linoleic in the oleic and linoleic acids
Outer back				
shoulder	30·4	73·1	5·3	22·1
central	33·9	72·9	7·6	19·5
tail	35·9	69·6	7·0	23·8
Inner back				
shoulder	37·6	69·6	8·3	25·0
central	42·9	66·4	10·6	23·6
tail	43·4	68·9	13·3	24·3
Leaf (perinephric)	51·0	64·7	16·9	27·7

high stearic content of most animal depôt fats is attained by a process of saturation of oleic, rather than of linoleic, derivatives.

The amounts of linoleic acid in the mixed fatty acids are in all cases larger than in any of the fats discussed in Part I of this series, as will be seen from Table V.

Table V. *Molar content of linoleic acid in the total fatty acids of various pig fats.*

Diet of pigs	Outer back	Inner back	Peri-nephric
Control (*ca.* 1·5 % fat)	9	8	5*
,, ,, +3 % Shea fat	10	7·5	3·5*
,, ,, +3 % arachis oil	13	11	10
Containing *ca.* 7 % fish meal	15	13	13

* Possibly slightly low, owing to rancidity.

In consequence of these proportions, and of the fact that oleic acid diminishes as stearic acid increases, the ratio of linoleic to oleic acid increases in the group now being described from the outer back fats to the leaf fat (*i.e.* as unsaturation decreases); this ratio was almost constant in all three fats from pigs whose diet had included arachis oil, and increased in the opposite sense (from the leaf fat to the outer back fat) in the remaining cases in which the diet contained relatively saturated, or no added, fat. We are thus led to believe that the relative amounts of these two unsaturated acids in any pig fat depend merely upon (i) the amount of stearic acid formation which has

occurred, coupled with (ii) the amount of linoleic acid present in the mixed fatty acids as a whole; the latter depends to a large extent on the diet and increases, but within comparatively narrow limits, from the perinephric to the outer layers of the back fats.

The occurrence of 1–2 % of unsaturated acids of the C_{20} and C_{22} series in all the depôt fats of a pig fed on a diet including fish-meal is noteworthy, since these proportions are definitely greater than those (up to 0·4 %) which Brown and Deck [1930] have shown to be characteristic of normal lards; the details of typical ester fractionations given in Table II illustrate this clearly, and also show that in the pig fats dealt with in the first part of this series there is a slight tendency to increase in iodine value and saponification equivalent in the residual esters, which is consistent with the presence of traces of arachidonic acid as postulated by these investigators.

The diet of the animal which yielded the fats now under discussion contained on an average 7 % of fish meal, and may thus have included about 0·7 % of fish oils; the latter will have contained about 20 % of the highly unsaturated C_{20} and C_{22} acids and an equal or perhaps somewhat greater amount of linoleic acid. Whilst we lack, unfortunately, precise data for the fat intake in the diet and the total amount of storage fat in the animal, it appears that the 1–2 % of unsaturated C_{20} and C_{22} acids in the latter might represent at least an appreciable proportion of the total quantity of these in the ingested fish meal; but, on the other hand, the recent observations of Brown [1931] go to show that lard from pigs fed on a diet which included 14 % of menhaden oil only contained 2·7 % of C_{20} and C_{22} acids, which were slightly less unsaturated than the corresponding acids of the oil itself.

It seems, then, that (as in the case of vegetable oils) the characteristic acids of ingested animal fat appear in pig depôt fats, but that the C_{20} and C_{22} acids are not stored so freely as oleic and linoleic acids; the latter, indeed, is laid down in unusually large amounts, although the amount present in the fish oil would not greatly have exceeded that of the C_{20} and C_{22} derivatives. This ready assimilation of linoleic acid into the present series of pig depôt fats is in keeping with our previous observations on fats from pigs fed on a diet including arachis oil, and with those of Ellis and Isbell [1926], who showed that, in extreme cases, the linoleic acid contents of pig fats almost reproduced those of the vegetable oils (arachis or soya bean) which formed the main part of the diet.

These results, on the whole, corroborate the general trade experience that a fish meal diet leads to the production of extremely soft fat: the softness is due both to general increase in unsaturated components and, especially, to unusually large proportions of linoleic acid. The C_{20} and C_{22} acids are not present in sufficient quantity to add appreciably to the soft qualities of the fats, but on the other hand might well, with the onset of slight rancidity, be responsible for development of a fish-like taint in the flavour.

Component glycerides of pig depôt fats.

The fully-saturated glycerides of the central outer and inner back layers, and of the perinephric fat from the animal whose diet was described on p. 298 were isolated quantitatively by our usual procedure (oxidation of unsaturated components by means of potassium permanganate in acetone), as described in detail by Hilditch and Sleightholme [1931]. In addition, two of the nine pig fats, the fatty acids of which were recently described by Bhattacharya and Hilditch [1931], were also submitted to this process (the quantity available of the remaining seven fats was insufficient for glyceride structure determination): the specimens utilised were perinephric fats from pigs (i) fed on a control diet and (ii) fed on the control diet + 3 % of arachis oil.

The numerical details of the isolation and purification of fully-saturated glycerides are collected in Table VI: it will be observed that the small pro-

Table VI. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) Analytical data.

			Purification of crude fully-saturated glycerides							
				"A"		"B"		Acidic products		
	Weight taken g.	Crude F.S.G. g.	Weight taken g.	g.	Acid No.	g.	Acid No.	g.	Acid No.	F.S.G. % (wt.)
Outer back	809	21·4	20·9	13·7	0·3	3·3	5·4	3·9	172·3	2·1
Inner back	806	57·1	54·6	45·4	0·4	5·7	6·8	3·5	278·2	6·6
Perinephric	402	49·5	47·9	38·9	0·3	4·9	5·5	4·1	238·4	11·2
Perinephric (previous series, control diet)	148	27·4	26·5	18·7	0·5	6·4	5·2	1·4	186·7	17·4
Perinephric (previous series, control + 3 % arachis)	130	19·7	18·8	11·5	0·5	4·7	3·8	2·6	170·8	13·0

(ii) Summary of observed values.

	Iodine value	Total saturated acid content % (mol.)	Fully-saturated glycerides M.P. °C.	Fully-saturated glycerides Weight %	Fully-saturated glycerides Mols. %	"Association ratio" in non-fully-saturated part. Mols. saturated acid per mol. unsaturated acid
Outer back	72·6	33·9	?	2·1	2·2	0·48
Inner back	64·6	42·9	60·5	6·6	6·7	0·63
Perinephric	59·0	51·0	60·5	11·2	11·4	0·81
Perinephric (previous series, control diet)	45·7	55·1	53·0	17·4	17·7	0·83
Perinephric (previous series, control + 3 % arachis)	55·1	46·9	53·5	13·0	13·2	0·64

portions of fully-saturated glycerides in the back fats necessitated the oxidation of very large amounts of the latter in order to obtain sufficient material for the subsequent determination of their component fatty acids by ester fractionation.

The purified fully-saturated glycerides "A" (Table VI) were hydrolysed and the resulting acids converted into mixed methyl esters, which were frac-

tionally distilled in the usual manner in order to determine their composition. It may suffice to record the final results obtained (Table VII).

Table VII. *Component fatty acids of fully-saturated glycerides of pig fats.*

	Weight percentages			Molar percentages		
	Myristic %	Palmitic %	Stearic %	Myristic %	Palmitic %	Stearic %
Outer back	0·3	52·2	47·5	0·3	54·7	45·0
Inner back	1·4	55·1	43·5	1·6	57·5	40·9
Perinephric	1·0	55·6	43·4	1·1	58·0	40·9
Perinephric (previous series, control diet)	1·6	59·3	39·1	1·9	61·5	36·6
Perinephric (previous series, control + 3 % arachis)	2·2	46·0	51·8	2·5	48·4	49·1

The relationship between the molar percentage of fully-saturated glycerides and the total molar content of saturated acids in these pig fats is very similar to that which was observed in the cases of tallows [Banks and Hilditch, 1931] and of butter fats [Hilditch and Sleightholme, 1931]: indeed, when plotted graphically (Fig. 1) the whole series, ranging from the outer back pig fat (34 % saturated acids, 2 % F.S.G.) to a butter fat containing 72·5 % saturated acids and 41·5 % fully-saturated glycerides, lies on a fairly smooth curve.

The composition of the fully-saturated glycerides is not quite so regular as that of the similar portions of tallows and milk fats, the molar content of stearic acid lying between the extreme limits of 37 and 49 %. In the three fats from different parts of the same animal, however, this figure varies only from 41 to 45 %. On the whole the ratio of palmitic (with myristic) to stearic acid in the fully-saturated components in these pig depôt fats is very similar to that in the corresponding glycerides of tallows. The content of myristic acid is distinctly lower than in tallow fully-saturated glycerides and, correspondingly, the melting-points (60·5°) of the fully-saturated components of the pig fats containing 41 mols. % of stearic acid were observed to be several degrees higher than those (54–54·5°) of tallow fully-saturated glycerides of similar stearic acid content.

Some features of the deposition of storage fats suggested by their structure.

The analyses reported, in this and preceding papers, of depôt fats of pigs and cattle (and also, to a certain extent, those of cow milk fats) disclose a number of consistent similarities which will demand attention when the manner in which storage fats are laid down is considered.

The most arresting feature is the circumstance that, whatever the degree of saturation of a depôt fat, its molar content of C_{18} acids is in the neighbourhood of 70 % (rising to about 73 % with very unsaturated fats, and falling to about 65 % in the more saturated fats). The actual degree of saturation is controlled, almost wholly, by the relative amounts of stearic and oleic acids present in any given case.

If it were assumed, firstly [*cf.* previous papers, 1931, pp. 1180, 1960], that the primary phase of the glycerides which finally appear as depôt fat is that of a comparatively unsaturated mixture produced by lipoclastic esterification of a mixture consisting, for example, of about 30 mols. of palmitic (including subordinate amounts of myristic) with about 70 mols. of oleic (with linoleic and probably subordinate amounts of stearic) acid, and secondly, that such initially-formed glycerides subsequently undergo hydrogenation (*i.e.* act as acceptors of hydrogen, in conjunction with some independent oxidation process proceeding in the same location), the observed approximate constancy of the C_{18} acid content in all normal depôt fats so far studied by ourselves and by other workers would follow as a matter of course. It is suggestive, moreover, to note that hydrogenation of the oleo-derivatives of preformed glycerides (but not necessarily of oleic acid prior to conversion into mixed glycerides) would inevitably produce a series of fats in which the relations between fully-saturated glyceride content and total saturation would be of the nature which we have found so uniformly characteristic for animal fats and, equally, so different from those of practically all the seed fats studied up to the present.

The observed tendency for the fully-saturated glycerides of depôt fats to possess similar compositions (frequently in the region of 60 mols. of palmitic to 40 mols. of stearic acid) is also in consonance with the supposition that glyceride hydrogenation may be an essential factor in the sequence of changes leading to storage fat. Within the limits of saturation concerned in any of the animal fats we have examined, the composition of fully-saturated materials would be kept within a comparatively narrow range, because dipalmito-oleins (with only one unsaturated acid group) will attain, *ceteris paribus*, complete saturation more readily than palmitodioleins, and still more so than triolein.

The glyceride structure of cotton-seed oil after partial catalytic hydrogenation has recently been investigated by Hilditch and E. C. Jones [1932] and the results are perhaps worth comparing with those for animal fats, since cotton-seed oil resembles our hypothetical "initially synthesised" animal glyceride mixture in so far as it contains about 25 % of saturated (nearly all palmitic) and 75 % of unsaturated acids, with no fully-saturated glycerides. During hydrogenation in presence of nickel at 180° the first action is almost selective conversion of linoleic to oleic (and *iso*-oleic) glycerides, and little increase in stearic (and consequently negligible formation of fully-saturated) glycerides takes place; but, from the point at which linoleic derivatives have disappeared the process is chemically not dissimilar (except in temperature conditions) from that which we have suggested as operative in the animal. It is interesting to observe that the relationship between fully-saturated glyceride content and degree of saturation of the hydrogenated cotton-seed oils, which is illustrated in Fig. 1, follows throughout the similar values which have been traced in this and preceding papers for pig fats, tallows and butter fats; the coincidence, although not complete, is distinctly more close than that with the curve for glycerides synthesised in the laboratory or with the mathematically-

derived graph, especially for fats of very small fully-saturated glyceride content. It should be added, moreover, that both in hydrogenated cotton-seed and hydrogenated olive oil there was marked selective hydrogenation of palmito-glycerides before tristearin was produced in noticeable amount. We propose to undertake similar hydrogenations of one of the more unsaturated pig fats and to examine the glyceride structure of resulting products, comparable in degree of saturation with some of the more saturated lards and tallows which we have investigated.

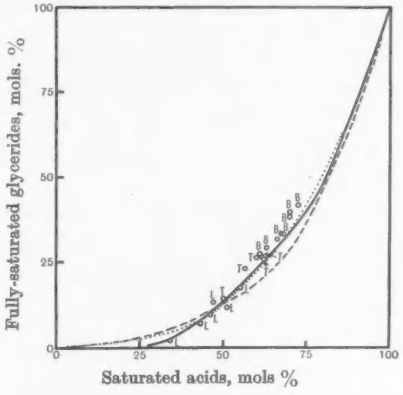

Fig. 1.

——— Hydrogenated cotton-seed oil
– – – – Synthesised glycerides
..... $y = kx^3$

B Cow butter fats
T Cattle body fats
L Pig body fats

It is, we believe, sometimes overlooked that the occurrence of stearic acid as a major component (*e.g.* forming 10 % or more) of natural fats is practically confined to non-aquatic mammals; it rarely forms more than about 1 % of marine animal oils or of vegetable fats, except in the case of the members of a very few tropical families (Sapotaceae, Guttiferae, Sterculiaceae, Dipterocarpaceae). Its relative abundance in animals may be a consequence of its derivation from preformed oleic glycerides in order to produce the more saturated fats of higher melting-point.

Whilst the composition of storage fats thus leads us to favour the view that a hydrogenation of glycerides rather than of free fatty acids is an essential link in the process of their formation, it clearly leaves us unable to suggest with any confidence where this change may take place. In view of the evidence disclosed in Part I of this series [1931], including the results obtained by Ellis and co-workers [1930, 1931], we believe that the characteristic constancy at about 70 mols. % of total C_{18} acids in pig depôt fats is most closely maintained when the diet of the animal has only contained small proportions of fat, and accordingly this may point to fat synthesised in the animal primarily from carbo-

hydrate as being the main seat of the suggested hydrogenation process; but this does not preclude the possibility that directly assimilated fat may also be affected. On the other hand, however, the evidence of Ellis and Zeller [1930] that linoleic acid in pig depôt fat is derived exclusively from assimilated vegetable linoleic acid may indicate that assimilated fat from the diet does not undergo hydrogenation to any material extent.

The tendency observed, in the less saturated depôt fats of a pig, for the component fatty acids to include somewhat more than 70 mols. % of C_{18} acids, and, conversely, for the C_{18} acid molar content to fall below this figure in the more saturated depôt fats from the same animal, would be explicable if specific mixtures of glycerides were selectively withdrawn from the blood at different sites of deposition. This would presuppose the presence in the blood of a common stock of glycerides in which the chemical processes had been completed, a possibility which is perhaps less remote than the alternative of hydrogenation to varying degrees in the adipose tissues themselves.

We wish to thank Captain Golding for assistance in obtaining the fats with which we have dealt with in this paper, and the Department of Scientific and Industrial Research for a grant to one of us (A. B.) during the course of the work.

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XXXV. PERMEABILITY OF VITAL MEMBRANES.

THE RED BLOOD CORPUSCLE.

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The red blood corpuscle possesses a remarkable property of retaining potassium as 99 % of its cations [Kramer and Tisdall, 1922], whilst the proportion of potassium in the surrounding serum is extremely low; at the same time the exchange of oxygen and some other substances is practically unhindered. In the study of vital membranes it was thought that the red blood corpuscle, being so easily obtainable, might afford indications of the passage of therapeutic substances and foodstuffs through other vital membranes. A considerable amount of work has already been done on this subject. Höber [1912] found that the property of permeability of the corpuscular envelope to ammonium was altered by various conditions, of special interest being his work with toxic substances. Noguchi [1905] showed that cobra venom under certain conditions made the corpuscles absolutely impermeable even to distilled water. Winokuroff [1929] found that partially haemolysed corpuscles, reverted by saline, had lost their selective permeability to anions and cations.

Nicloux and Yovanovitch [1925] determined the distribution of ethylene to be about 75 % in the corpuscles and 25 % in the plasma.

Methods.

In the tests which follow, the concentrations of substances in solution were determined before and after contact with a known volume of washed corpuscles. We first of all found that the erythrocyte showed absolute impermeability to sulphates and ready permeability to bromides, and by utilising a solution of known strength of bromide and sulphate *plus* substance to be tested, the errors consequent upon large volumes of quasi-solid material in the mixtures could be eliminated. From a theoretical point of view one need not even know the initial volume of blood corpuscles, the figures being obtainable by mathematical calculation from the proportions of sulphate and bromide before and after the test.

Sheep's blood corpuscles were washed several times by centrifuging with saline and the volume of corpuscles, after centrifuging for half an hour, was measured. A solution of the substance to be tested, adjusted to p_H 7·4, was added in the proportion of 1 of solution to 3 of corpuscles. After thorough mixing during half an hour the mixture was again centrifuged and the supernatant solution analysed.

By the use of masses of raw egg-white suspended by wire hooks in a solution, we were able to investigate selective permeability of albuminous masses without any intervening lipoid envelope. Ten minutes in a solution containing equal proportions of bromide and sulphate resulted in penetration of five times as much bromide as sulphate. The difference in velocities of the ions in water being insignificant there was thus a real selective permeability.

In view of the highly albuminous nature of the red blood corpuscle this property may be of great importance in addition to the lipoid envelope. Both factors taken together constitute an ideal condition resembling a vital membrane with its adjacent protoplasm elsewhere in the body.

Results.

(1) Inorganic.

A. *Cations.*

(*a*) *Potassium.* It may be taken without further experimental proof that the erythrocyte is impermeable to potassium.

(*b*) *Ammonium.* Determination of NH_3 was made using three salts—chloride, bromide and sulphate. In each case ammonium was found to penetrate readily, attaining equal proportions with the solute in a short time (Table I, Exps. 1, 2, 3).

(*c*) *Copper.* Copper tartrate was used; contact of solution with corpuscles for over 1 hour resulted in only slight diminution of copper, the amount not exceeding the probable surface absorption (Table I, Exp. 4).

(*d*) *Calcium.* $CaCl_2$ used; contact for 1 hour resulted in practically no passage of calcium. (In this experiment it was necessary to lower the p_H to 7·2 (Table I, Exp. 5).) Leiboff [1930] found that calcium is entirely absent from the corpuscles of human blood.

B. *Anions.*

(*a*) *Sulphate.* Used in the form of salts of sodium, potassium and ammonium. No permeation of the corpuscle occurred (Table I, Exp. 6 and others).

(*b*) *Chloride.* Used in the form of sodium and ammonium salts. The corpuscles were readily permeable to chloride ion (Table I, Exps. 7 and 8).

(*c*) *Bromide.* Employed as sodium and ammonium salts. With ammonium bromide it was necessary to reduce the time of contact with the blood to avoid haemolysis, and to make the p_H of the solution 6·8 (2 % disodium hydrogen

phosphate, p_H 6·0, did not cause haemolysis of the same specimen of blood) (Table I, Exps. 9*a*, 9*b*, 9*c*).

(*d*) *Dichromate.* As ammonium and potassium salts. There was a definite penetration of the corpuscle, which became brown owing to the formation of

Table I. *Permeability of red blood corpuscles for inorganic ions.*

(Single salts.)

Experiment	Ion	Salt	Initial concentration of salt %	Concentration of ion in corpuscles % / Concentration of ion in liquid %	Permeability, *i.e.* (quotient × 100)
1	NH_4	NH_4Cl	4·0	0·42 / 0·42	100
2	NH_4	NH_4Br	4·0	0·36 / 0·36	100
3	NH_4	$(NH_4)_2SO_4$	2·0	0·18 / 0·19	95
4	Cu	Cu tartrate	3·0 Cu	0·4 / 2·6	15
5	Ca	$CaCl_2$	31 mg. Ca per cc.	0·6 / 19·4 mg. per cc.	3
6	SO_4	Na_2SO_4, $10H_2O$	2·81	0·04 / 0·77	5
7	Cl	NaCl	1·5	0·31 / 0·32	99
8	Cl	NH_4Cl	4·0	0·46 / 1·5	30
9*a*	Br	NaBr	1·5	0·58 / 0·58	100
9*b*	Br	NaBr	2·5	0·66 / 0·66	100
9*c*	Br	NH_4Br	4·0	1·06 / 1·06	100
10*a*	Cr_2O_7	$K_2Cr_2O_7$	5·0	1·23 / 1·19	100
10*b**	Cr_2O_7	$K_2Cr_2O_7$	2·75	0·59 / 0·84	70
11	Cr_2O_7	$(NH_4)_2Cr_2O_7$	0·42	0·104 / 0·139	75
12	$Fe(CN)_6$	$K_4Fe(CN)_6$	5·0	0·20 / 2·8	7
13*a*	$(COO)_2$	$(COOK)_2$	4·0	0·29 / 1·33	22
13*b*	$(COO)_2$	$(COOK)_2$	13·0	†1·76 / 3·8	46

* $N/20$ dichromate added = 55·2 cc. $N/20$ dichromate removed in supernatant liquid = 26·4 cc. Dichromate removed from corpuscles by washing 4 times with 5 cc. of saline = 26·5 cc. Total dichromate recovered = 52·9 cc.

In this Exp. the corpuscles, after exposure to the dichromate, contained only 10 % of oxyhaemoglobin, the remainder being converted to methaemoglobin.

† Expressed as oxalic acid.

methaemoglobin within an apparently intact membrane. After centrifuging it was found that, by washing with saline, the dichromate which had penetrated the corpuscles could be recovered (Table I, Exps. 10 and 11).

(*e*) *Ferrocyanide.* Used as potassium ferrocyanide. No penetration occurred (Table I, Exp. 12).

(*f*) *Oxalate.* Used in the form of a 4 % solution of potassium oxalate; also in stronger concentration of about 13 % after neutralisation by titrating with oxalic acid solution to p_H 7·2. A definite decrease in the concentration of oxalate of surrounding solute was observed (Table I, Exp. 13) indicating some penetration.

(*g*) *Ferricyanide.* Used as potassium ferricyanide. No penetration occurred.

The test with ferricyanide solution thus showed marked contrast with the dichromate in that no change in the constitution of the haemoglobin occurred whilst protected by the corpuscular envelope. The oxygen capacity of the blood corpuscles (determined according to Woodhouse and Pickworth [1930]) was unaffected even by contact with 8 % potassium ferricyanide. Similar analyses of blood corpuscles after contact with $N/2$ potassium dichromate showed that 90 % of the oxyhaemoglobin was converted to methaemoglobin.

Experiments using $N/20$ potassium permanganate and varying concentrations of potassium mercuric iodide failed, owing to the destructive action on the corpuscles, which were quickly haemolysed, the haemoglobin being converted to methaemoglobin.

(*h*) *Bromide and sulphate.* Washed corpuscles were mixed with 1·5 % sodium bromide + 2·8 % sodium sulphate. After a suitable period of contact and centrifuging, analysis showed that the bromide had penetrated readily and the sulphate not at all (Table II B, Exp. 1).

(*i*) *Bromide, sulphate and dichromate.* A mixture containing 3 % sodium sulphate, 2 % sodium bromide and 2·5 % potassium dichromate was left in contact with the corpuscles for half an hour.

The penetrations of the ions in this experiment were equivalent to those which took place in absence of the other salts. Although the haemoglobin was largely converted to methaemoglobin, the bromide penetrated readily and the sulphate not at all. The selective properties of the membrane were not affected even although complete alteration in the chemical constitution of the protein within the corpuscle occurred (Table II B, Exp. 2).

(2) Organic.

Dyes.

The distribution of a number of water-soluble dyes between corpuscles and the liquid phase was studied by estimating the amount of colour remaining in solution before and after contact with the washed corpuscles for 30 minutes. In most cases the Lovibond tintometer was employed but in a

few cases comparison was made in a Klett colorimeter. Various concentrations of dye were tried.

The dyes employed fell into 4 groups.

(1) Impermeable (permeability quotient 0–2·6)—trypan red, trypan blue, naphthol green, eosin, erythrosin, phenol red, acriflavine, Congo red (0·05 %).

(2) Semi-permeable (permeability quotient 5–10)—naphthol black, orcein, safranine, carmine, neutral red.

(3) Permeable (permeability quotient 33–100)—acid fuchsin, pyronin, rosolic acid, toluidine blue, Bismarck brown, nigrosine, Congo red (0·5 %).

Table II. *Permeability of red blood corpuscles.*

Experiment	Ion tested	Compound	Initial concentration %	Concentration in corpuscles % / Concentration in liquid %	Permeability (*i.e.* quotient × 100)
(A) Organic compounds.					
1*a*	—	Urea in isotonic saline	10	3·48 / 5·5	63
1*b*	—	,,	5	1·65 / 2·62	63
1*c*	—	,,	1	0·30 / 0·50	60
1*d*	—	,,	1	0·20 / 0·33	61
2*a*	—	Glucose	5	1·08 / 2·85	38
2*b*	—	,,	2	0·45 / 1·10	41
(B) Inorganic mixtures.					
1	Br	Sodium bromide 1·5 % +	0·98	0·48 / 0·49	100
	SO_4	Na_2SO_4, $10H_2O$ 2·8 %	0·85	0·0077 / 0·68	1·1
2	Br	Sodium bromide 2·0 % +	1·30	0·606 / 0·56	100
	SO_4	Na_2SO_4, $10H_2O$ 3 % +	0·89	0·030 / 0·63	5
	Cr_2O_7	Potassium dichromate 2·5 %	1·69	1·12 / 1·32	85
(C) Mixed organic and inorganic substances.					
1	SO_4	Na_2SO_4, $10H_2O$	3·0	0·0465 / 0·88	5
	Urea	Urea	1·0	0·29 / 0·50	58
2	Glucose	Glucose (pure)	2·0	0·44 / 1·14	39
	Bromide	Sodium bromide	2·0	0·52 / 0·54	100
	Sulphate	Na_2SO_4, $10H_2O$	3·0	0·040 / 0·83	5

(4) Indeterminate (adsorption or chemical combination making estimation of permeability inaccurate)—china blue, wasser blau, Nile blue sulphate, methylene blue, janus green, gentian violet, thionine blue, victoria blue, brasilin, brilliant green, dahlia, carminic acid, Biebrich scarlet.

Organic compounds of physiological importance.

Urea. It was found that over a wide range of concentrations of urea the final ratio of urea in corpuscles to fluid was 1·0/1·6. The initial concentrations used and the final values are given in Table IIA.

Urea and sulphate. A solution of 1 % urea and 3 % sodium sulphate was employed. It was found that the urea penetrated easily and the sulphate radical remained entirely outside the corpuscle (Table IIC, Exp. 1). (Lundsgaard and Holbøll [1926] found that fructose, galactose, acetone and urea all distributed themselves between plasma and corpuscles in the ratio of 1·4/1. Duval [1923] also found the blood corpuscles to be easily permeable to urea and dimethylurea.)

Glucose. Solutions of 5 % aqueous glucose and 2 % glucose in saline were employed. Contact with corpuscles caused a marked decrease in concentration of the glucose. (The glycolytic action of the blood was controlled by another experiment with the addition of sodium fluoride.) An easy penetration of the corpuscles by glucose occurred (see Table IIA, Exp. 2).

Glucose + bromide + sulphate. Glucose and bromide each readily entered the corpuscles, being unaffected by the other constituents of the mixtures. Sulphate again remained entirely outside the corpuscles (see Table IIC, Exp. 2).

PHYSICOCHEMICAL ALTERATIONS IN THE CONDITION OF LIPOID ENVELOPE OF CORPUSCLE.

It has been demonstrated (Table IIB, Exp. 2) that conversion of haemoglobin to methaemoglobin by dichromate did not alter the permeability of the cells to the other substances used. It is therefore of practical importance to know the effect of certain substances of physiological or therapeutic interest which presumably act on the envelope.

The following were substances employed: (1) formaldehyde, (2) saponin, (3) veronal, (4) streptococcus toxin.

(1) In concentration of 1–2 % neutralised formaldehyde saline caused the washed corpuscles to become a gelatinous, compact mass after exposing for 3 minutes. Haemolysis was not observed. With 0·4 % solution of formaldehyde there were no changes observed in the structure and no apparent difference from normal corpuscles in the relative permeabilities of bromide and sulphate.

(2) The quantity of saponin added was 0·5 cc. of 0·1 % solution in saline to 5 cc. of washed corpuscles, contact being allowed for 30 minutes. The corpuscles were then again washed with saline and tested with bromide-sulphate

mixture. No measurable change was found to have occurred in the permeability of bromide or sulphate.

(3) Veronal[1] and (4) streptococcus toxin (non-haemolytic) similarly gave no appreciable changes in the permeability in the concentration used.

Semi-permeability of protein gel (raw egg-albumin).

20 cc. egg-white were placed in a collodion bag which was suspended in 20 cc. of a mixture of sulphate 1·4 % and bromide 0·75 %. After 20 hours, the liquid was analysed with the following result: sulphate 0·73 %, bromide 0·42 %. Thus both sulphate and bromide penetrated to very similar extents. Since this test gave no indication of the relative speeds with which the equilibrium with respect to the two ions was attained, and as the collodion membrane introduced a factor of uncertainty, experiments were devised to avoid this by using masses of raw egg-white in direct contact with the solution.

Direct exposure of egg-white to salt solutions.

Small compact masses of egg-white were suspended in tubes containing sulphate and bromide. After intervals of 10–60 minutes these were withdrawn, the supernatant liquid removed by blotting-paper and the content of bromide and sulphate determined in the albuminous mass.

The weight of the specimen being determined, the albumin was coagulated by boiling with a few cc. of water acidified with dilute acetic acid, the clear liquid filtered, made up to 10 cc. and the bromide and sulphate estimated in the usual manner. A control analysis was made by adding 0·5 g. of egg-white to 0·5 cc. of the solution of salts and carrying out the analyses as above.

The results are given in Table III.

Table III. *Permeation of raw egg-albumin.*

Time in minutes	Sulphate (per g. of albumin) mg.	Bromide (per g. of albumin) mg.	$\frac{\text{Bromide}}{\text{Sulphate}}$	Permeability ratio: $\frac{\text{Bromide}}{\text{Sulphate}}$
10	1·8	3·61	2·0	5·0
10	2·9	3·36	1·2	3·0
12	3·2	5·28	1·7	4·1
15	11·4	8·02	0·7	1·8
15	11·2	8·10	0·73	1·8
60	15·4	8·57	0·56	1·4
60	15·3	8·28	0·54	1·4
Control	24·3	9·8	0·40	1·0
Original solution	1 cc. = 10 mg. NaBr = 25 mg. Na_2SO_4, $10H_2O$			

Thus the bromide in 10 minutes had penetrated 5 times as much as the sulphate. After 1 hour, 85 % of the bromide, but only 62 % of the sulphate

[1] Fabre and Fredet [1925] after intravenous injections of veronal found the ratio

$$\frac{\text{veronal in corpuscles}}{\text{veronal in serum}} = \frac{1{\cdot}88}{1{\cdot}00}.$$

had passed into the albumin. The difference is too great to be accounted for entirely by the relative mobilities of the individual ions and shows that the protein content of the corpuscle envelope controls to a marked extent the entry or exclusion of ions.

Differential permeation through egg-white.

A glass cell $3'' \times 1\frac{1}{2}''$ was divided into three compartments by two parchment membranes fixed to the sides by wax so that direct flow of liquid from section to section could not occur. 15 cc. of egg-white were placed in the central portion and bromide + sulphate solution was placed in one end, section *A*, and distilled water in the other, *B*.

After 12 hours, bromide had passed into the central compartment but none appeared in section *B*. After 30 hours, bromide appeared in compartment *B* and the following ratios were found:

$$\frac{\text{Bromide in } B}{\text{Bromide in } A} = \frac{1}{2{\cdot}5},$$

$$\frac{\text{Sulphate in } B}{\text{Sulphate in } A} = \frac{1}{16}.$$

Thus bromide had preferentially migrated, confirming the results obtained in the preceding experiments.

Summary.

The permeability of the red corpuscle to various inorganic ions, dyes and organic compounds of physiological importance has been studied.

By analogy with the permeability of raw egg-white it is shown that the protein as well as the lipoid envelope exerts a selective permeability to salts.

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XXXVI. THE MECHANISM OF THE FORMATION OF ORGANIC ACIDS BY MOULD FUNGI.

I. THE ACTION OF *ASPERGILLUS NIGER* ON SODIUM HEXOSEDIPHOSPHATE IN THE PRESENCE OF TOLUENE.

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(*Received January 28th, 1932.*)

Examination of the various hypotheses which have been formulated in explanation of the mycological conversion of hexoses into citric acid shows that the chemical changes involved in this transformation have been regarded from at least four different points of view.

Kostytschew and Tschesnokow [1927] advanced the opinion that citric acid is a by-product of protein synthesis from sugar. Raistrick and Clark [1919] considered it possible that a molecule of glucose might by loss of two molecules of water and oxidation at the terminal carbon atoms yield β:δ-diketoadipic acid which, on hydrolysis, should split readily into acetic and oxaloacetic acids. In the view of these authors an aldol condensation between the two latter acids might then be presumed to yield citric acid.

Now the formation of citric acid by mould fungi is usually accompanied or followed by the formation of oxalic acid, and the suggestions of Raistrick and Clark provided a reasonable explanation of this fact, since they supposed oxalic acid to be derived from acetic acid by oxidation and from oxaloacetic acid by hydrolytic fission to acetic acid followed by oxidation. It has been suggested that citric acid might arise *via* compounds of 3 carbon atoms formed by preliminary fission of the sugar molecule [*cf.* Euler, 1909; Amelung, 1927; Chrząszcz and Tiukow, 1930]. Other views have involved suggestions that the hexose chain may be converted into a molecule of citric acid by intramolecular change preceded or followed by processes of dehydration and/or oxidation [*cf.* Knoop and Windaus, 1905; Buchner and Wüstenfeld, 1909; Butkewitsch, 1924; Franzen and Schmitt, 1925; Challenger, Subramaniam and Walker, 1927].

The last named authors, employing a single strain of *A. niger*, obtained small yields of saccharic acid from cultures on glucose and on calcium gluconate, and also detected citric acid in cultures of this mould on potassium saccharate media. On this evidence and in view of the observation of Franzen and Schmitt [1925] that β:γ-diketoadipic acid on treatment with alkali metal

hydroxides undergoes a "benzilic acid transformation" to citric acid, the suggestion was made that the conversion of glucose to citric acid by *A. niger* might proceed by the stages:

gluconic acid → saccharic acid → β:γ-diketoadipic acid → citric acid.

The subsequent work of one of us with other strains of *A. niger* has not, however, supported this view, nor has it been found possible to detect the presence of β:γ-diketoadipic acid in cultures of the original strain of *A. niger* on glucose and on saccharic acid.

In view of these facts and, in particular, in view of the recent failure of Bernhauer, Siebenäuger and Tschinkel [1931] to obtain evidence of citric acid formation in cultures of numerous strains of *A. niger* on saccharic acid or potassium saccharate media, it must be concluded that the conversion of saccharic acid into citric acid in the single case mentioned above is to be regarded as a strain characteristic, and is not of general significance in the scheme of reactions leading to the formation of citric acid.

On the grounds that those species of *Citromyces* and of *Aspergillus* which are typical producers of citric acid give rise simultaneously to little or no ethyl alcohol, whilst, on the other hand, those *Mucor* species, *e.g. M. racemosus*, which form appreciable amounts of ethyl alcohol from sugar are incapable of also giving as end-products more than traces of acids, Butkewitsch [1923] concluded that the processes leading to citric acid formation cannot be considered to be closely related to those which give rise to ethyl alcohol.

Bernhauer and Schön [1928] were also inclined to the same opinion since they were unable to "trap" more than traces of acetaldehyde in numerous experiments in which strains of *A. niger*, known to be capable of forming citric acid, were cultivated on glucose, fructose and glycerol [*cf.* Neuberg and Cohen, 1921].

Recently, however, Chrząszcz and Tiukow [1930] established the facts that the action of *Penicillium citrogenum* on sodium acetate affords fumaric, *l*-malic and citric acids, and that cultures of the same mould on potassium acetate give rise to the formation of succinic, *l*-malic, citric and oxalic acids.

More recently still, Bernhauer and Siebenäuger [1931] have been able to reproduce these results by the employment of a number of strains of *A. niger*. The latter authors have also obtained citric acid by the action of *A. niger* on ethyl alcohol in the presence of sodium bicarbonate or of calcium carbonate, and Chrząszcz and Tiukow (private communication) have observed the formation of citric acid from the same source, thus confirming a much earlier observation of Mazé and Perrier [1904] which had hitherto lacked substantiation.

These results rendered necessary a reconsideration of the question as to whether compounds of 3 carbon atoms, derived from sugars, are precursors of citric acid, and Chrząszcz and Tiukow expressed the view that the transformation of glucose into citric acid occurs through reactions which may be divided into two phases, in the first of which the stages are presumed to be: hexose → a hexosephosphoric ester → methylglyoxal → pyruvic acid → acetaldehyde → alcohol, and in the second of which it is supposed that acetalde-

hyde and ethyl alcohol give acetic acid → succinic acid → fumaric acid → *l*-malic acid. This last acid is then presumed to condense with acetic acid (by dehydrogenation) yielding citric acid. This assumption as to the course of the reactions in the first phase is supported by the fact that Molliard [1922] and Raistrick and his collaborators [1931] have identified glycerol among the metabolic products from glucose of two different species of *Aspergillus*, whilst the view as to the course of the reactions in the second phase receives support (1) from the experiments of Butkewitsch and Fedoroff [1930], which demonstrated the formation of succinic and fumaric acids when *Mucor stolonifer* was allowed to act upon calcium acetate or upon ethyl alcohol in the presence of calcium carbonate, (2) from the observation of Challenger and Klein [1929] that the action of a strain of *A. niger* on fumaric acid afforded a high yield of *l*-malic acid, and (3) from the work of Stent, Subramaniam and Walker [1929] which established the fact that *A. niger* converts succinic acid *via* fumaric acid into *l*-malic acid.

Whilst there is thus strong experimental evidence in favour of Chrząszcz and Tiukow's explanation of the formation of citric acid from acetic acid, the production of methylglyoxal by mould fungi has not hitherto been established. It might be claimed that the formation of acetaldehyde and of ethyl alcohol, which has been observed during the development of various moulds on sugar media, constitutes indirect evidence of a prior formation of methylglyoxal but, on the other hand, it might also be argued that acetaldehyde and ethyl alcohol could be derived from oxaloacetic acid produced from glucose according to the mechanism suggested by Raistrick and Clark.

The experiments of Quastel [1924] with bacteria, of Stent, Subramaniam and Walker [1929] with *A. niger*, and of Hahn, Haarmann and Fischbach [1929] with muscle provide ample evidence of the extreme readiness with which oxaloacetic acid, in the presence of certain biological agents, undergoes decarboxylation to pyruvic acid. Further, Nagayama [1921] and Walker and Coppock [1928] have shown that the development of various moulds, including *A. niger*, on calcium pyruvate, affords acetaldehyde and ethyl alcohol.

Accordingly, the present authors have commenced a further study of the reactions which give rise to citric acid, and in this communication it is shown that the action of *A. niger* on sodium hexosediphosphate in the presence of toluene leads to the formation of methylglyoxal in quantity sufficient to permit of its isolation as the 2:4-dinitrophenylosazone.

Experimental.

Neuberg and Kobel [1930] have shown that in order to demonstrate the formation of methylglyoxal by the desmolysis of sugar under the influence of yeast, it is not essential to carry out preliminary liquefaction of pressed yeast by plasmolytic agents. The desired inhibition or weakening of the co-zymase can be effected equally well in the presence of magnesium hexosediphosphate

which, in aqueous solution, is shaken with fresh "top-fermentation" yeast together with toluene or other suitable plasmolytic agent. On now allowing the mixture to stand at 37° methylglyoxal and pyruvic acid are produced and accumulate in the medium.

Our experiments with *A. niger* were designed, so far as possible, on similar lines. In preliminary work use was made of boiling-tubes each containing 15 cc. of a 5 % solution of glucose together with inorganic salts (NH_4NO_3 0·2 %; KH_2PO_4 0·1 %; $MgSO_4,7H_2O$ 0·05 %).

After sterilisation these were given heavy sowings of spores and were incubated for various lengths of time; after which the media were removed by sterile pipettes and sterilised tap-water at 30° was added in the same manner, the tubes being then returned to the incubator for 3 or 4 hours.

At the end of this time the water was removed and a second treatment with sterile water was given, followed by a further period of incubation for 3 hours.

The second wash-water was now withdrawn as before, and solutions of sodium hexosediphosphate containing 1 % of toluene were added. The tubes were again placed in the incubator at 30° and, after various intervals of time, samples were withdrawn and tested for the presence of methylglyoxal by means of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride in 2*N* hydrochloric acid (1200 cc. contained 4 g. of the base).

Six strains of *A. niger*, all of which are known to be capable of citric acid formation, were employed in the preliminary experiments, and are designated N2, B8, B16, No. 3528·7, W16 and W17. Of these, N2 was supplied by Prof. C. Neuberg, B8 and B16 were received together with other strains from Dr K. Bernhauer of Prague and No. 3528·7 was sent to us by Dr C. Thom of the United States Department of Agriculture. We have pleasure in expressing our indebtedness to these gentlemen for their assistance. Strains W16 and W17 were isolated by one of us from plates of suitable agar medium which had been exposed to the open air.

The sodium hexosediphosphate employed was highly purified material supplied by I. G. Farbenindustrie A.G. (Elberfeld), and some experiments were also undertaken with a pure specimen of calcium hexosediphosphate which was made available through the courtesy of Dr F. F. Nord.

The data obtained from a large series of small-scale experiments showed that dilute solutions (from 0·25 to 1 %) of sodium hexosediphosphate gave larger percentage yields of what, presumably, was methylglyoxal, than were obtained when higher concentrations of the salt were employed, and that it was advantageous to use young cultures of the mould before sporulation was far advanced. The optimum yields of the 2 : 4-dinitrophenylosazone were obtained when the reagent was added to the hexosediphosphate solutions 24 hours after they had been placed in contact with the mould.

Precipitates of the osazone were obtained from experiments with each of the six *A. niger* strains. These precipitates melted at about 250° but after they had been washed separately with 2*N* hydrochloric acid, water, 20 % sodium

carbonate solution, and water, in that order, their melting points were raised to about 280°. On treatment with alcoholic potassium hydroxide solution they all gave rise to the intense blue-violet coloration described by Neuberg and Kobel as characteristic of the 2 : 4-dinitrophenylosazone of methylglyoxal.

In further experiments calcium hexosediphosphate was employed. Owing to its slight solubility this salt could not be used in concentrations higher than approximately 0·4 to 0·5 %, but such solutions gave yields approximating to those obtained from the use of 1 % solutions of the sodium salt.

In order to accumulate sufficient precipitate for purification and analysis a series of experiments was set up in flasks. In one of these experiments, the details of which are cited below, three concentrations of sodium hexosediphosphate were adopted, in order to provide quantitative data as to the yields.

Ten 300 cc. Erlenmeyer flasks, each containing 100 cc. of a 5 % solution of glucose together with the necessary salts, were heavily inoculated with spores of strain W16 and incubated at 30° for 2 days. The media were then removed, the mycelia washed as already described, and the flasks were divided into three groups (A), (B) and (C), to which were then added 1 %, 0·5 % and 0·25 % aqueous solutions of sodium hexosediphosphate, respectively, together in each case with 1 % of toluene. The liquid was removed from the mycelia after contact for 24 hours at 30° and the filtered solutions were combined in their respective groups (A), (B) and (C), and each was treated with 500 cc. of 2 : 4-dinitrophenylhydrazine hydrochloride solution.

After standing for 2 hours the resulting precipitates were filtered off, washed as described above, dried and weighed. Each precipitate melted at 280°. The yields of methylglyoxal calculated on the actual weights of sodium hexosediphosphate used in the several cases were as follows.

Group (A). From 1 % hexosediphosphate, equivalent to 5 %.
Group (B). From 0·5 % ,, ,, ,, 13 %.
Group (C). From 0·25 % ,, ,, ,, 16 %.

The sodium carbonate washings in no case gave more than traces of deposit when acidified with hydrochloric acid, hence pyruvic acid 2 : 4-dinitrophenylhydrazone was not present in appreciable amounts.

In this experiment "control" flasks were also set up in which mycelia were allowed to stand in contact with (*a*) water and (*b*) a 1 % solution of sodium hexosediphosphate, in both cases without toluene, and (*c*) water *plus* 1 % of toluene. In no case did subsequent addition of 2 : 4-dinitrophenylhydrazine hydrochloride solution cause either formation of a precipitate or clouding of the liquid. It should also be remarked that precautions were adopted, throughout the course of these experiments, to exclude the possibility of bacterial contamination of the cultures or of the hexosediphosphate solutions. Careful microscopical examination and sub-culturing failed to detect bacterial contamination in any single experiment.

The precipitates from (A), (B) and (C) were combined (0·47 g.) and re-

crystallised twice from nitrobenzene at 150–160°, when, after washing with alcohol and ether and drying at 100°, the melting-point was 298°. (Found, by micro-analysis, N 25·5 %; 26·03 %; calculated, N 25·93 %.)

Starting from glycerol a specimen of the 2 : 4-dinitrophenylosazone of methylglyoxal was prepared by the method of Neuberg and Hofmann [1930]. After purification this melted at 298° and a mixture of the two specimens also melted at the same temperature.

Summary.

It has been found that by suitable adaptation of the method whereby Neuberg and Kobel demonstrated the formation of methylglyoxal by the action of yeast on sodium hexosediphosphate it is possible to show that the latter substance affords methylglyoxal when submitted to the action of *Aspergillus niger* in the presence of toluene. The product was characterised as the 2 : 4-dinitrophenylosazone. This behaviour of the mould is in harmony with the view of Chrząszcz and Tiukow that the early stages of the transformation of glucose into citric acid by mould fungi involve the formation of a hexose-phosphate and its conversion into methylglyoxal.

Our best thanks are due to Mr H. Shaw who carried out the micro-analyses.

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XXXVII. THE INFLUENCE OF THE MEDIUM ON THE PRODUCTION OF BACTERIAL GELATINASE.

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In a previous communication [Haines, 1931] it was shown that bacterial proteases are produced by organisms growing in simple synthetic media made up from carefully purified substances, and the influence of salts of calcium and magnesium on growth and protease formation was discussed. The methods used to estimate protease formation, namely, liquefaction of gelatin as evidenced by inability to set in ice-water and lack of coagulation of caseinogen on addition of acid, were however too crude to yield quantitative data as to the relative efficiency of the media in stimulating or inhibiting enzyme formation. In particular, it is to be expected that small amounts of gelatinase will be formed on certain media, insufficient in quantity to cause entire liquefaction, which will escape detection by such a method. More sensitive ways of measurement were therefore sought, and changes in viscosity of a mixture of gelatin and enzyme seemed to offer a ready means of following gelatinase action. Manning [1924] showed that the viscosity of 5 % gelatin could be measured in the ordinary way in an Ostwald viscometer provided that the determinations were carried out at 35° or above. Below 35° the gelatin is in gel form and possesses appreciable rigidity, but at 35° and above it is a sol. The shape of the viscosity-time curve also depends on the previous treatment of the gelatin, but is reproducible if the gelatin be made up under standard conditions.

Experimental.

The organisms used were freshly isolated from dung. In the previous work [1931] the strains of *Proteus* obtained would not grow in synthetic media. One strain has subsequently been isolated, however, which grew well, and the experiments described in this paper have been conducted with it and a strain of *Pseudomonas*. The characteristics of these organisms are listed in Appendix I. In general the organisms were grown on nutrient agar, p_H 7·4, overnight at 20°, the surface growth was scraped off, and washed and centrifuged in sterile saline three times. 1 cc. of the final suspension was used for inoculation purposes. The routine technique was the same as described in the previous paper, all substances being purified in a similar manner and carefully cleaned

pyrex glass-ware and ammonia-free re-distilled water used throughout. All media were made up by dilution from separate sterile solutions of their constituents. The gelatin was 5 % Gold Label in phosphate buffer of the required p_H, never more than half an hour's heating on the water-bath at not above 60° being employed. 0·5 % phenol was added and the filtered product stored in 12 cc. lots in tubes closed with corks coated with tin-foil. Cotton-wool had to be dispensed with owing to the trouble caused by small fibres finding their way into the viscometer. Blank viscosity determinations with a mixture of sterile medium and gelatin gave the type of curve shown in Fig. 1, constant for some hours and then decreasing slightly. To test gelatinase formation, the medium in which the organism had been grown was centrifuged at high speed for three quarters of an hour. By this means nearly all the organisms were removed, and the necessity of filtration through a Chamberland candle, experimentally tedious when a number of media are to be compared simultaneously, was avoided. Test experiments showed that the mixture of centrifuged medium and phenol-gelatin remained sterile. Comparison between filtered and unfiltered centrifugates also indicated that the few, if any, organisms remaining in the unfiltered preparations caused no increase in the digestion of the gelatin. In general, 5 cc. of the centrifugate were added to 10 cc. melted phenol-gelatin, both having been previously brought to 37° in the incubator, and 10 cc. of the well shaken mixture pipetted immediately into a clean dry Ostwald viscometer suspended in a water-bath at 37° ± 0·1°. A few minutes were allowed for the mixture to regain a temperature of 37°, because trial experiments showed that although both solutions were at 37° prior to mixing, and pipettes and viscometers had been stored at that temperature before use, a lowering of 2–3° was inevitable during mixing. A first reading was then taken and others at half-hour intervals. Duplicate readings usually agreed to a fraction of a second, but the readings were taken to the nearest second. In the case of a very active broth centrifugate, however, the change at the beginning was so rapid that duplication of the initial reading was impossible with viscometers giving readings of the order of 90–150 seconds. Comparative experiments with several viscometers however gave identical results. All readings are expressed as percentages of the initial reading, thus cancelling variation from viscometer to viscometer. Relative rate of change of viscosity only has been measured and not absolute viscosity. Using four different viscometers and the same centrifugate, the four sets of figures nowhere differed by more than 2 %, and a change of viscosity of the order of 50 % is being measured with a broth culture. This is shown in Fig. 1, where the time for the rate of fall of the gelatin-enzyme mixture between the graduations on the viscometer, expressed as a percentage of the initial reading, is plotted against time of incubation. The control readings with gelatin and sterile broth are also shown.

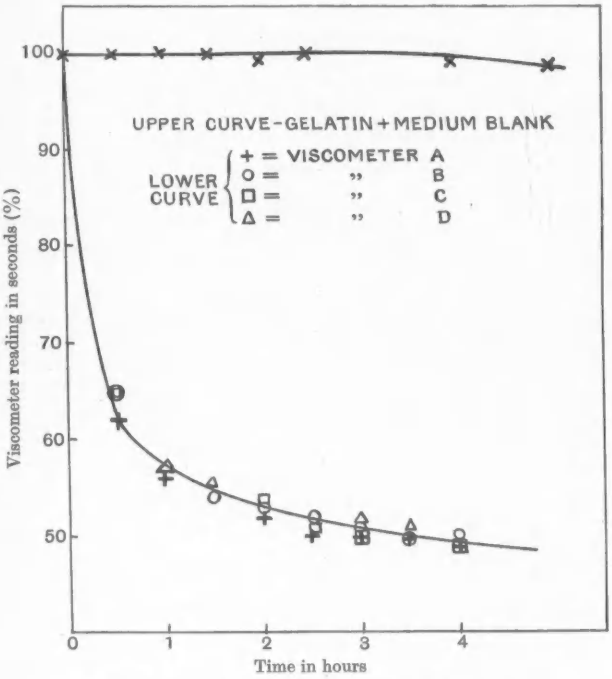

Fig. 1. Comparison of viscometers and broth control.

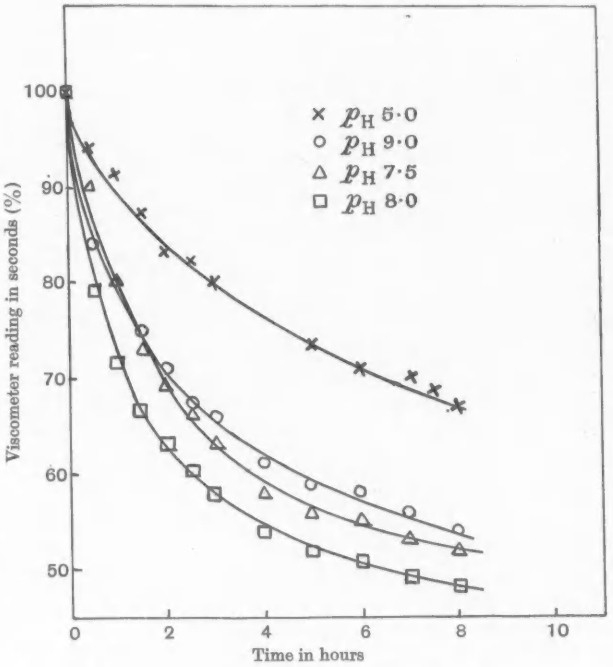

Fig. 2. Effect of p_H.

Gelatinase formation in broth.

Preliminary experiments were first carried out with the organism growing in nutrient broth, p_H 7·4, in order to follow the influence on gelatinase production of such factors as the age of the culture and the p_H of the digestion mixture.

The optimum p_H *for gelatinase action.* Batches of phenol-gelatin were made up in suitable buffers of p_H 5, 7·5, 8 and 9 approximately, checked by the colorimetric method. The centrifugate from a 94-hour culture of the *Proteus* organism was then added to each and the change in viscosity determined. The results are shown graphically in Fig. 2. It will be seen that the greatest change took place at p_H 8 and the least at p_H 5. The optimum p_H for the action of the gelatinase is thus 8, and all subsequent digestions were carried out at that p_H. This finding is in agreement with that of Merrill and Clark [1928] who also worked with *Proteus* bacteria and estimated the gelatinase activity by the time required for setting under standard conditions.

The age of the culture. 500 cc. of nutrient broth p_H 7·4 were inoculated with a single loopful of the *Proteus* organism in a litre pyrex flask and incubated at 20° with continued shaking. From time to time samples were withdrawn and gelatinase activity estimated, during the period 2–38 days. Plate counts of the rate of multiplication of the organisms were also carried out. The curves representing gelatinase activity are shown in Fig. 3. In order to obtain a numerical basis for comparison, the time for a 25 % change in viscosity has been found from each curve, and the reciprocal of this multiplied by ten, together with the corresponding plate counts, is shown in Table I. A similar experiment was carried out with the *Pseudomonas*, except that in this case 1 cc. of the suspension of the washed organism was inoculated into the broth.

Table I. *Growth and gelatinase formation in broth.*

t = time for 25 % change in viscosity.

Proteus				*Pseudomonas*			
Time in hrs.	Plate count Orgs./cc.	Time in hrs.	Gelatinase $1/t \times 10$	Time in hrs.	Plate count Orgs./cc.	Time in hrs.	Gelatinase $1/t \times 10$
0	95,000	46	3	0	$1{\cdot}7 \times 10^6$	20	<0·5
22	230×10^6	70	7	24	60×10^6	42	8
42	1060×10^6	94	14	49	1300×10^6	90	30
66	1400×10^6	118	14	73	4700×10^6	113	50
115	4200×10^6	142	20	97	7600×10^6	136	50
167	4600×10^6	214	25	119	5200×10^6	184	58
		360	33				
		574	33				
		912	33				

It will be seen that with the *Proteus* the gelatinase activity of the centrifugate increased rapidly up to about 4 days, after which it increased slowly until a limit was reached at about 14 days. The numbers of organisms, on the other hand, did not increase greatly after about 5 days. From 14 to 38 days

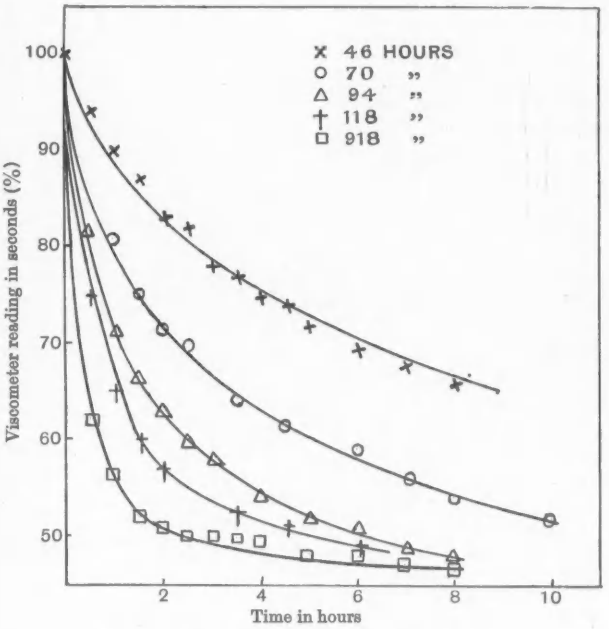

Fig. 3. Effect of age of culture. Broth. *Proteus.*

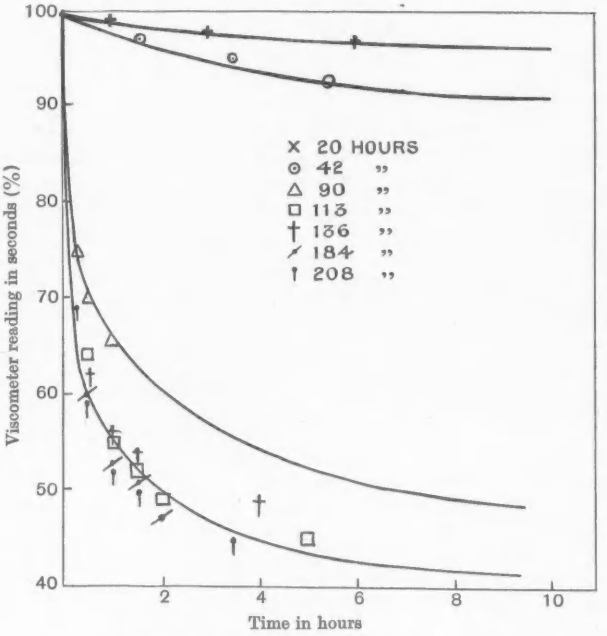

Fig. 4. Effect of age of culture. Clark's medium. *Pseudomonas.*

the gelatinase activity remained approximately constant, after which no further measurements were made. The rate of multiplication of the *Pseudomonas* was of a similar order, but a more active gelatinase was obtained than from the former organism, and it was apparently formed somewhat more rapidly.

Gelatinase formation in synthetic media.

The media of Merrill and Clark and a slight modification of that of Fränkel, as listed in the previous paper, have been used, except that sodium lactate was substituted for ammonium lactate in Fränkel's medium. A modified Fränkel's medium in which glycerol was substituted for sodium lactate was found to yield as good growth as the original medium and was considerably less troublesome to prepare. This medium is referred to as Fränkel A, and it had the following composition:

10 cc. 4 % glycerol.
10 cc. 1 % solution of pure amino-acid.
2 cc. 2 % KH_2PO_4.
2 cc. 5 % NaCl.

p_H adjusted to 7·4 with NaOH, and 1 cc. of 0·1 % $MgSO_4,7H_2O$ and 1 cc. of a suspension of 0·1 % $CaCO_3$ added where required.

Gelatinase formation has been measured in these media using varying sources of nitrogen. As a rule the first subculture and some subsequent one such as the third or sixth in any given medium have been tested, for there seems to be a general tendency for slightly more rapid growth and rather better gelatinase formation with repeated subculture. It is unlikely, therefore, that despite the washing with saline, anything has been carried over from the original agar culture, because in that case gelatinase activity would be expected to be greater at first and then to decrease on subculture. While it was not possible to carry out plate counts in every case, care has been taken to avoid misinterpretations of gelatinase activity owing to poor growth, the final growth having been estimated by turbidity measurements against $BaSO_4$ standards, made up in the manner described by Peskett [1927].

Gelatinase formation in Merrill and Clark's medium.

Gelatinase formation in Merrill and Clark's simple medium with NH_4Cl as the source of nitrogen and with calcium and magnesium salts present was first followed. 1 cc. of a three times washed suspension of the *Pseudomonas* organism in saline was added to 250 cc. medium in a 500 cc. Erlenmeyer pyrex flask, and approximately 10 cc. lots were withdrawn at intervals and gelatinase activity was estimated. Control experiments were carried out with gelatin *plus* sterile medium containing Ca and Mg salts. The curves so obtained were similar to that for gelatin *plus* sterile broth shown in Fig. 1, and are shown in Figs. 5 and 6. Plate counts were also carried out. These counts are shown in Table II together with the turbidity measurements and the figure obtained for 25 % change in viscosity. The complete curves are shown in Fig. 4.

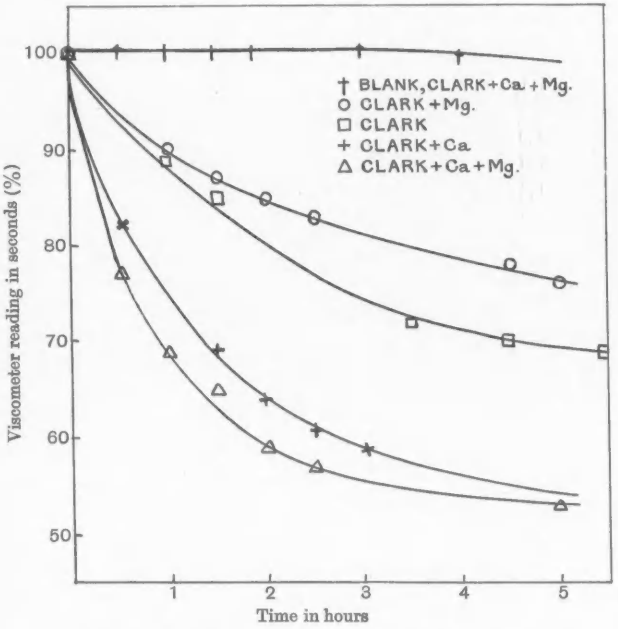

Fig. 5. Gelatinase production in Merrill and Clark's medium. First subculture 6 days. *Proteus.*

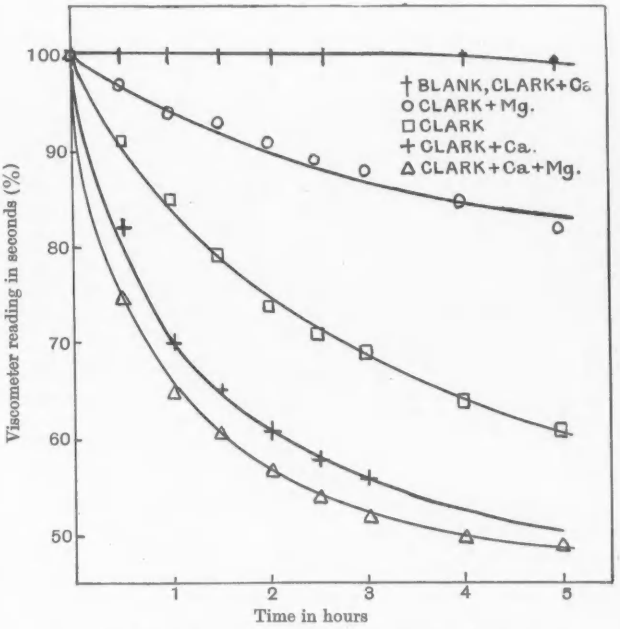

Fig. 6. Gelatinase production in Merrill and Clark's medium. Third subculture 9 days. *Proteus.*

Table II. *Gelatinase formation in Merrill and Clark's medium at 20° with* Pseudomonas, *in the presence of Ca and Mg salts.*

Time in hrs.	Plate count Orgs./cc.	Turbidity	Time in hrs.	Gelatinase $1/t \times 10$
0	$2{\cdot}5 \times 10^6$	$< M/1000$	20	$<0{\cdot}5$
24	8×10^6	$< M/1000$	42	$<0{\cdot}5$
49	71×10^6	$M/400$	66	36
73	520×10^6	$M/320$	90	36
97	870×10^6	$M/200$	113	40
119	1700×10^6	$M/80$	136	44
			184	52
			208	52

Turbidity was measured in terms of molar K_2SO_4 used in making up the standards. Thus $M/80$ signifies that one-eightieth molar K_2SO_4 solution was added to excess $BaCl_2$ solution. 10 cc. lots were used in sealed comparator tubes [see Peskett, 1927]. The method is not accurate with these bacteria owing to the difficulty of comparison between the fine bacterial suspension and the more coarsely granular $BaSO_4$. It served, however, as a useful basis of comparison, more accurate than the loose terms "good" or "poor" growth. Approximate calibration is provided for *Pseudomonas* by the figures of Table II; results with *Proteus* were of a similar order.

It will be seen that both growth and production of gelatinase, though slower in the synthetic medium, were eventually of a similar order to those obtained in broth culture. Preliminary experiments with both *Proteus* and *Pseudomonas* organisms in the presence and absence of salts of calcium and magnesium indicated that although greater production of gelatinase took place in the appropriate medium with *Pseudomonas*, yet this organism was more rigid in its requirements than *Proteus*. The formation of gelatinase in the absence of calcium and magnesium salts with the former organism, using NH_4Cl or certain amino-acids, was very much less than with *Proteus*, growth also being very poor. *Proteus* gave measurable amounts of gelatinase and fair growth with a wide range of sources of nitrogen, while the *Pseudomonas* seemed definitely to yield better results with the simplest medium containing NH_4Cl. For that reason *Proteus* was chosen as the more interesting organism for the majority of the experiments, and in the following examples it is to be understood that that organism is referred to unless it is expressly stated otherwise.

The influence of salts of calcium and magnesium on gelatinase production in Merrill and Clark's medium.

The results obtained from centrifugates of Merrill and Clark's medium with both calcium and magnesium salts present, and with each salt separately and neither salt, in the first and third subcultures of the *Proteus* are shown graphically in Figs. 5 and 6. The numerical figures in terms of 25 % change in viscosity, and the growth as estimated by turbidity, are given in Table IV for first, third and sixth subcultures. It will be seen that good growth and the

best gelatinase production occurred in the presence of both salts. Nearly as good gelatinase production was obtained with calcium salts alone, although the growth was much less. With magnesium salts alone growth was as good as with both calcium and magnesium and much better than with calcium salts alone or with neither, and yet gelatinase production was much less than with the former and appreciably less than with the latter. In order to clinch this point, counts were carried out in a similar manner to those of Table II, with *Pseudomonas* instead of *Proteus* as used above, growing in Marrill and Clark's medium with magnesium salts only, so as to make the figures comparable. The results are shown in Table III.

Table III. *Growth and gelatinase formation in Merrill and Clark's medium at 20° with* Pseudomonas, *in the presence of Mg salts only.*

Time in hrs.	Plate coun. Orgs./cc.	Time in hrs.	Gelatinase
0	10×10^6	22	<0·5
19	57×10^6	172	<0·5
72	1020×10^6	264	<0·5

It will be seen that the rate of multiplication is essentially similar in the two media, but gelatinase formation very much less in the latter one. Magnesium salts therefore have a stimulating influence on growth but an inhibitory action

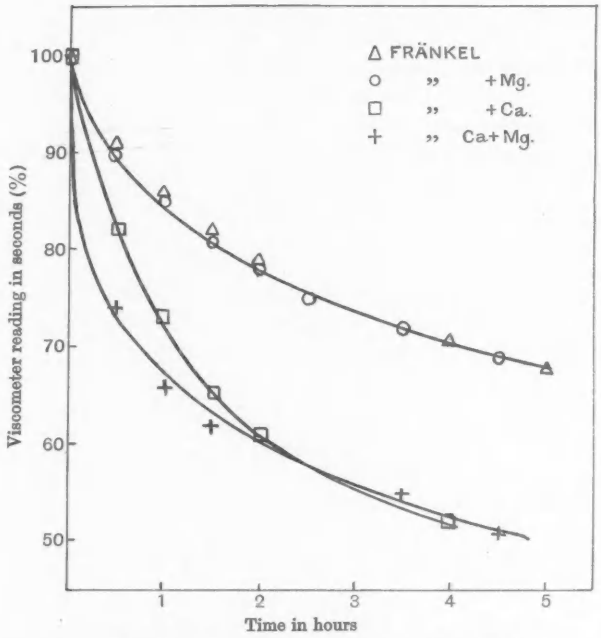

Fig. 7. Gelatinase production in Fränkel's medium. *Proteus.*

on gelatinase formation. Calcium salts have no stimulating action on growth but stimulate gelatinase production. The two salts together stimulate both growth and gelatinase production. It appears that the salt content of the medium is of much greater importance in determining the gelatinase production than the extent of growth of the organisms. While slight variation occurred in the results from subculture to subculture they are essentially similar in every case.

Gelatinase production in Fränkel's medium.

Fig. 7 represents the results of an experiment in Fränkel's medium with and without added salts. In this case salts of magnesium again stimulate growth (see Table IV) but although multiplication of the organisms is much

Table IV. *Gelatinase activity of the centrifugate, obtained from viscosity curves, $= 1/t \times 10$ where $t =$ time for 25 % change.*

Proteus organism.

Medium	Source of nitrogen	Added salts	No. of subculture	Age of subculture days	Growth	Gelatinase $1/t \times 10$
Clark	NH_4Cl	None	1	6	*M*/400	4
,,	,,	Mg	1	6	*M*/80	2
,,	,,	Ca	1	6	*M*/800	12
,,	,,	Ca + Mg	1	6	*M*/80	17
,,	,,	None	3	9	*M*/800	5
,,	,,	Mg	3	9	*M*/80	1
,,	,,	Ca + Mg	3	9	*M*/80	20
,,	,,	Ca	3	6	*M*/800	14
,,	,,	None	6	11	*M*/800	3
,,	,,	Mg	6	11	> *M*/80	1
,,	,,	Ca	6	10	*M*/800	14
,,	,,	Ca + Mg	6	11	> *M*/80	13
Fränkel	Asparagine	None	1	7	*M*/400	4
,,	,,	Mg	1	7	*M*/80	4
,,	,,	Ca	1	7	*M*/400	12
,,	,,	Ca + Mg	1	7	*M*/80	22
,,	,,	None	4	9	*M*/200	5
,,	,,	Mg	4	7	*M*/80	6
,,	,,	Ca	5	9	*M*/80	17
,,	,,	Ca + Mg	5	9	*M*/80	18
Fränkel A	Glycine	None	1	8	*M*/400	3
,,	,,	Mg	1	7	*M*/80	3
,,	,,	Ca	1	5	*M*/200	2
,,	,,	Ca + Mg	3	7	*M*/80	14
,,	,,	None	3	7	*M*/400	6
,,	,,	Mg	3	6	*M*/320	1
,,	,,	Ca	3	6	*M*/400	2
,,	,,	Mg	2	5	*M*/80	3
,,	,,	Ca + Mg	4	10	*M*/80	17
,,	Alanine	None	1	8	*M*/400	1
,,	,,	,,	3	7	*M*/400	5
,,	,,	Ca + Mg	1	4	*M*/80	17
,,	,,	Ca + Mg	3	17	*M*/80	25
,,	Arginine	None	1	6	*M*/400	<0·5
,,	,,	Ca + Mg	1	4	*M*/200	6
,,	,,	Ca + Mg	3	13	*M*/80	13
,,	Glutamic acid	None	1	6	*M*/200	3
,,	,,	Ca + Mg	1	4	*M*/80	14
,,	Tyrosine	None	1	8	?*	<0·5
,,	,,	Ca + Mg	1	7	?	2

* It was impossible to estimate the turbidity in the presence of tyrosine owing to undissolved substance, but growth was slight.

Table IV (*cont.*).

Pseudomonas organism.

Medium	Source of nitrogen	Added salts	No. of subculture	Age of subculture days	Growth	Gelatinase $1/t \times 10$
Clark	NH_4Cl	None	1	4	*M*/1000	<0·5
,,	,,	Mg	1	4	*M*/80	<0·5
,,	,,	Ca	1	4	*M*/1000	<0·5
,,	,,	Ca + Mg	1	4	*M*/80	33
,,	,,	None	2	19	*M*/800	<0·5
,,	,,	Ca + Mg	2	19	> *M*/80	33
,,	,,	None	3	7	*M*/1000	<0·5
,,	,,	Mg	3	7	*M*/80	<0·5
,,	,,	Ca	3	7	*M*/1000	<0·5
,,	,,	Ca + Mg	4	8	> *M*/80	35
Fränkel A	Asparagine	None	1	3	*M*/800	<0·5
,,	,,	Ca	1	3	*M*/800	<0·5
,,	,,	Mg	1	3	*M*/80	<0·5
,,	,,	Ca + Mg	1	3	*M*/200	4
,,	,,	None	3	7	*M*/1000	<0·5
,,	,,	Ca	3	7	*M*/1000	<0·5
,,	,,	Mg	3	7	*M*/80	<0·5
,,	,,	Ca + Mg	3	7	*M*/80	10
,,	Glycine	None	1	4	*M*/1000	<0·5
,,	,,	Ca	1	4	*M*/1000	<0·5
,,	,,	Mg	1	4	*M*/200	<0·5
,,	,,	Ca + Mg	1	4	*M*/200	10
,,	,,	None	3	6	*M*/1000	<0·5
,,	,,	Ca	3	6	*M*/1000	<0·5
,,	,,	Mg	3	6	> *M*/80	<0·5
,,	,,	Ca + Mg	3	6	*M*/80	28

greater in Fränkel's medium *plus* magnesium salts than in this medium alone, gelatinase formation is almost identical in the two cases. With calcium salts growth was much less than with either magnesium, or calcium *plus* magnesium salts, yet gelatinase production was greater than in the unaltered medium or medium *plus* magnesium salts, and nearly the same as in the medium with both salts. Similar results were obtained from subsequent subcultures, and these are given in Table IV.

In Fränkel's medium, therefore, magnesium salts stimulate growth but have no action, either stimulative or inhibitive, on gelatinase production, but salts of calcium, while not affecting growth, stimulate the formation of gelatinase. As in other media, the greatest amount of gelatinase is yielded in the presence of both salts.

The production of gelatinase in other media.

Other sources of nitrogen have been substituted for asparagine in the medium Fränkel A and complete curves obtained representing a measure of the quantity of gelatinase produced. The figures found from these curves for 25 % change in viscosity of the gelatin-enzyme mixture are grouped together in Table IV.

Discussion.

It will be seen that a certain amount of gelatinase is detectable whatever the medium used, when sufficient growth has occurred. The setting-power of the gelatin is not a reliable criterion of gelatinase production, because unless sufficient enzyme has been formed to cause a change of 40–50 % in the viscosity the gelatin will still set when immersed in ice-water. It has been found that within this range the ability to set is somewhat indefinite. For example, a sample whose viscosity was 46 % of the original value set, while one with viscosity of 48 % of initial value did not. Possibly the added salts exerted an influence here. In a medium containing no added salts it makes little difference whether the source of nitrogen be ammonium chloride or any of the amino-acids used, except in so far as rather better growth is obtained with some amino-acids than others. The figures obtained for gelatinase production are not quite so good with tyrosine, arginine and alanine as with asparagine, glutamic acid, glycine and ammonium chloride, but a more accurate measure of growth than the turbidity tests would probably account for these relatively small differences. In every case, however, in a synthetic medium salts of calcium and magnesium together greatly stimulate formation of gelatinase, whatever the source of nitrogen. With ammonium chloride as the source of nitrogen, salts of magnesium alone seem partially to inhibit enzyme production, although at the same time stimulating growth, while salts of calcium alone, having no influence on growth, stimulate gelatinase formation. This effect is very marked, excellent growth with little gelatinase being obtained with the magnesium salts, and only medium or poor growth and good gelatinase with the calcium salts. Moreover, the production of enzyme in the medium containing the magnesium salt and giving excellent growth was less than that in the medium containing no added salts and giving moderate growth. While the factor of the amount of growth taking place must not be lost sight of, emphasis must be laid on the salt content of the medium as of fundamental importance in determining the amount of gelatinase occurring in it. The concentration of the salts causing these marked effects is of the order of 0·01–0·004 % as so far investigated; it remains to be seen what effect increasing this concentration would have. Also, while no marked diminution in the ability to produce enzyme in the synthetic medium with subculture has yet been noted, it must be remembered that the washed organism has so far been carried through only about six subcultures. Freshly isolated cultures seem to yield more active gelatinase preparations than those which have been kept on ordinary nutrient agar for some time, so that further experiment may show a similar effect in synthetic media. With asparagine as source of nitrogen magnesium salts stimulate growth but not enzyme production, while calcium salts again affect the latter without influencing the former. With glycine, however, neither calcium nor magnesium singly affects the gelatinase although magnesium salts here again stimulate growth, but the two salts combined exhibit their

usual effect. The reasons for the varying influence of the single salts and yet the almost constant effect of their combination, with different sources of nitrogen, are not at present apparent. Explanation must be sought, however, in some action on the bacterial cell during growth. An experiment was tried in which 0·1 cc. of the suspension of calcium carbonate was added to a centrifugate from a culture of Clark's medium *plus* magnesium salts. The curves obtained both with and without addition of calcium salts were identical and similar to the top curve of Fig. 6. That is, salts of calcium added subsequently had no activating influence on the gelatinase produced previously in the medium containing magnesium salts.

Summary.

1. By following the change in viscosity of a mixture of gelatin and sterile centrifugate from the medium in which the organism had been grown, a certain amount of gelatinase was demonstrated whatever the medium used, providing adequate growth occurred.

2. In a synthetic medium containing no magnesium or calcium salts, no single amino-acid was markedly superior to any other or to ammonium chloride in its effect on gelatinase production, except in so far as rather better growth was obtained with some than others.

3. In all cases salts of calcium and magnesium together greatly stimulate the production of gelatinase.

4. In some cases salts of magnesium alone, while stimulating growth, partly inhibit gelatinase formation. Salts of calcium alone have little or no growth-stimulating action but in most cases greatly stimulate gelatinase production.

5. In a medium containing ammonium chloride, glucose and phosphates, together with salts of calcium and magnesium, gelatinase is produced in amount comparable with that obtained in broth culture.

6. The explanation of the function of these salts must be sought in some action on the cells apart from stimulation of growth or activation of the enzyme.

The author wishes to express his indebtedness to Dr G. S. Graham-Smith for his interest and advice, and to Mr J. Barlow for assistance in the experimental work.

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APPENDIX. *Cultural characteristics of the organisms used.*

Proteus. *Motile Gram-negative rod*	Pseudomonas. *Motile Gram-negative rod*
Agar—Raised, smooth, white to cream	Moist, raised, cream growth, bright green fluorescence
Gelatin—Crater liquefaction, becoming stratiform	Crater liquefaction
Potato—Moist, raised, cream to brown	Spreading, dirty cream to brown
Litmus milk—Alkaline, peptonised	Alkaline, peptonised
Broth—Pellicle and sediment	Heavy turbidity
Nitrates—Reduced	Reduced
Indole—Doubtful	Not formed
Lactose—Not fermented	Not acid
Maltose—Acid and gas	Not acid
Sucrose—Acid and gas	Acid, no gas
Dextrose—Acid and gas	Acid, no gas
Mannitol—Acid and gas	Not acid
Galactose—Acid, no gas	Acid

XXXVIII. THE OXYTOCIC PRINCIPLE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND. I.

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Some controversy has taken place in the past as to the number of hormones responsible for the varied physiological activities of extracts of the posterior lobe of the pituitary gland. Engeland and Kutscher [1912] stated that the substance stimulating the contraction of uterine muscle (oxytocic activity) is not identical with that which raises arterial blood pressure (pressor activity), and Dudley [1919, 1923] and Kamm *et al.* [1928] presented conclusive evidence on this question, and obtained solid preparations which exhibited a high oxytocic activity whilst retaining relatively little pressor substance.

The work described in this communication has dealt solely with the oxytocic principle, and was designed to purify the substance responsible for this type of activity by extraction or adsorption from solutions which had been partially freed from inactive material. Considerable purification has been effected by adsorption of the active substance on norite charcoal, and on the basis of the results obtained it is hoped to investigate new methods of obtaining oxytocic preparations, more especially by the introduction of adsorption stages early in the process.

The experiments now recorded indicate that a study of the influence of hydrogen ion concentration on the adsorption of the oxytocic principle by highly purified charcoal [*cf.* Phelps and Peters, 1929; Phelps, 1931] may yield information as to the presence of acidic and basic groups. This will be the subject of a future communication.

Experimental.

The whole of the work has been carried out with a sample of the powdered dried posterior lobe of ox pituitary gland presented to us by Boots Pure Drug Company Limited, to whom we are deeply indebted.

In our experiments it was desirable to use an oxytocic preparation which was relatively free from inactive solids and pressor substance, and we selected as our starting material a partially purified extract prepared by the method of Kamm *et al.* The purification has not been carried farther than the "partial separation of the two active principles" described [Kamm *et al.*, 1928, p. 590]. Thus, in a successful extraction, estimations made with aliquot parts of

the extract showed that 100 g. of posterior lobe powder assaying 1·5 international oxytocic units (I.U.) per mg. yielded an aqueous solution containing 170 mg. of solid with an oxytocic activity of 30,000 units and a small pressor activity. In the following pages, a "Kamm" solution indicates one prepared by the method of the American workers and purified to the stage mentioned above.

Attempts to effect purification by butyl alcohol extraction.

Dudley [1919] observed that the oxytocic substance could be removed from dilute aqueous extracts of the posterior lobe by means of continuous extraction with butyl alcohol. Repetition of this work with partially purified extracts showed that the ratio of activity/solid diminished in the course of the experiment, and confirmed the loss of activity by decomposition as noted by Dudley.

The butyl alcohol was purified by distillation and distillation in steam, thus ensuring its complete volatility and removal after the experiment. The solid contents of the solutions were estimated by evaporating aliquot portions to constant weight at 100°.

Exp. 1. 20·0 cc. of a Kamm solution containing 68 mg. of solid and having an oxytocic activity of 12,000 I.U. were extracted for 11 hours, and the butyl alcoholic extract was evaporated to dryness under reduced pressure at 45°. The residual solid was taken up in water, brought to p_H 3 with pure acetic acid, and made up to 50·0 cc. This solution contained 50 mg. of solid and showed an oxytocic activity of 7500 units. The ratio of activity in units/mg. solid has thus fallen from 176 : 1 at the beginning of the experiment to 150 : 1 at the end.

Exp. 2. 30·0 cc. of a Kamm solution containing 102 mg. of solid and having an activity of 18,000 I.U. were treated as before. The resulting solution of the extract contained 85 mg. of solid with an activity of 7300–10,000 units, and the aqueous residue contained 20 mg. of solid and only 40 units. The whole of the solid is thus accounted for, but at least 8000 units have been destroyed. The ratio of activity/solid of the butyl alcoholic extract has fallen from 176 : 1 to 117 : 1 during the experiment.

During these experiments, it was observed that part of the solid is removed from the aqueous layer very rapidly, and appears as a precipitate in the alcoholic extract. Since the rate of extraction of the active principle has not been investigated, it is not as yet known if this fact points the way to a method of purification.

Purification by adsorption.

It was evident from the investigations and conclusions of others that adsorption was likely to prove of little benefit if the active substance could only be eluted by treatment with vigorous chemical reagents, such as mineral acids or, more especially, alkalis. Further, the ease with which the active substance is adsorbed on precipitates formed in its presence made it clear that adsorptions by precipitates were to be avoided, if the liberation of the active material involved the solution of the adsorbent and its subsequent reprecipitation at a later stage. We therefore selected norite charcoal as the adsorbent.

So far as we have been able to ascertain, there is no record of the behaviour of solutions of the oxytocic substance when exposed to charcoal. Sato [1928], it is true, has observed a great diminution of activity when a pituitary extract was shaken with charcoal, but his experiments were directed along other lines and have little bearing on the present investigation.

Purification of charcoal. 200 g. of norite charcoal were heated with 2 litres of boiling 10 % hydrochloric acid for 7 hours, filtered, washed thoroughly with water, and extracted with 1200 cc. of 6*N* hydrochloric acid in an open basin on a boiling water-bath for 18 hours. The charcoal was filtered, washed with 30 litres of hot water, stirred many times with distilled water, and collected on several wide Büchner funnels so that each funnel was covered with a layer of charcoal about 1–2 cm. in depth. The charcoal was then washed with distilled water until the washings were completely free from chloride, and was dried at 120° in an air-oven. Finally, small portions of the charcoal were heated at red heat in a silica flask for 40 minutes at a pressure of 1 mm. of mercury, each portion being allowed to cool to room temperature under the reduced pressure. These samples were thoroughly mixed and were preserved in a stoppered bottle in a desiccator. All the adsorption experiments recorded here have been carried out with this specimen of charcoal, the ash content of which was 0·7 %.

Preliminary adsorption experiments. A few tentative experiments convinced us that charcoal adsorption might be made to play an important part in the purification of the oxytocic substance, and offered some encouragement to proceed to a more elaborate scheme. The details of two typical examples of these preliminary experiments are briefly recorded below on account of their correspondence with the results obtained later with a different pituitary solution.

Exp. 3. At p_H 3·5. 22·0 cc. of a Kamm solution assaying 4900 I.U. were brought to p_H 3·5, mixed with a sodium acetate-acetic acid buffer at p_H 3·5 and with 700 mg. of purified norite. The mixture was left at room temperature (18–20°) for 22 hours in a closed flask, and shaken occasionally during that period. The charcoal was collected by filtration, washed with a little distilled water, and dried over phosphoric oxide. When dry, the charcoal was eluted with glacial acetic acid (20 cc.) at room temperature for 3 hours, filtered off, and washed with glacial acetic acid. The filtrate and washings were concentrated to 1–2 cc. under reduced pressure at 45°, diluted with water, and made up to 25·0 cc. at p_H 3. 1·0 cc. of this solution contained from 100 to 150 oxytocic units, so that the maximal recovery of activity was 3750 units, or about 75 % of the initial activity.

Exp. 4. At p_H 10. 2·3 cc. of a Kamm solution assaying 550 I.U. were brought to p_H 10 by the addition of sodium hydroxide, and were mixed with 2·3 cc. of 0·1*N* sodium phosphate buffer at p_H 10 and then with 100 mg. of purified norite. The mixture was left at room temperature for 3 hours, being occasionally shaken, and the charcoal was collected, washed with a little water, dried over phosphoric oxide, and eluted for 3 hours with glacial acetic acid as before.

The acetic acid solution was evaporated to dryness under reduced pressure at 45°, and the residue dissolved in water and made up to 10·0 cc. at p_H 3. This solution contained 50–60 units per cc., the total activity thus being 500–600 units.

The aqueous liquid from the adsorption was brought to p_H 3 with a volume of 10·0 cc. and then contained less than 5 units per cc. Thus less than 50 units failed to be adsorbed, and all the activity was accounted for between the buffer solution and the charcoal, on which 91 % of the active material was adsorbed.

These results were surprising at the time, in view of the well-known destruction of the oxytocic substance by cold alkalis [Guggenheim, 1914], but subsequently Gaddum [1930] prepared a nomogram from which it may be calculated that less than 1 % of the activity would be destroyed in this experiment assuming that the rate of destruction of the oxytocic substance is the same in partially purified solutions as in whole extracts of the posterior lobe. In this connection it may be mentioned that Gaddum [1930] found that purification did not affect stability at p_H 7 and 99°, and Gerlough and Bates [1930] state that the heat-stability of the oxytocic substance when partly freed from accompanying inactive material is unchanged as compared with that in posterior lobe extracts.

Adsorption between p_H *1 and 13.* Preliminary experiments, which were carried out with different Kamm solutions, indicated that comparable results of adsorptions at different hydrogen ion concentrations could only be obtained by the use of a uniform stock solution containing sufficient material for all the estimations.

250 g. of posterior lobe powder were extracted by the Kamm method, and the product was brought to p_H 3 by addition of pure acetic acid and made up to 100·0 cc. of aqueous solution, but without being buffered. This stock solution was therefore free from extraneous solids, and contained 2·5 mg. per cc. with a total oxytocic activity of 20,000 I.U. This value for the activity is low, a fact which may be attributed to the difficulty of manipulating large quantities during the purification, and of storing them under anhydrous conditions before use. The stock solution was preserved at 0° in a refrigerator, and was only removed for short periods to allow the withdrawal of samples. Its activity remained undiminished for 6 months—the duration of the series of experiments and assays.

All precautions were taken to ensure the elimination of extraneous solid matter from the glacial acetic acid and distilled water used, and volumes of these much greater than those needed for each adsorption were frequently examined for contamination. Further, a standard technique using the same apparatus was adopted for the adsorption experiments and was rigidly adhered to throughout the series of tests.

Buffering between p_H 3·8 and 5·7 was effected by means of 0·2 *N* sodium acetate-acetic acid solutions [Cole, 1928]. The adsorption at p_H 2·9 was carried out with an unbuffered 0·1 *N* acetic acid solution. At p_H 7–11, sodium

phosphate buffers were utilised [Prideaux, 1911]. Solutions at p_H 1, 2 and 13 were made by the addition of 0·1 *N* hydrochloric acid, 0·01 *N* hydrochloric acid or 0·1 *N* sodium hydroxide respectively, the volume added being the same as the amount of buffer added in the other experiments. Potassium salts were avoided in making up buffer solutions, since the potassium ion interferes with the oxytocic assay [Burn and Dale, 1922].

Procedure. 2·00 cc. of the stock solution were brought as nearly as possible to the required p_H by the addition of *N* or 0·1 *N* hydrochloric acid or sodium hydroxide solutions. This solution was cooled in ice and transferred by means of 6·0 cc. of ice-cold buffer solution into a wide, squat, glass-stoppered weighing bottle containing 100 mg. of purified norite. The norite was thus exposed in a thin layer to liquid about 1 cm. deep, and possible errors due to slow diffusion were reduced to a minimum. The weighing bottle and contents were then placed in a refrigerator at 0° for exactly 5 hours, being gently agitated at hourly intervals. At the same time, a control experiment was carried out (see below).

At the end of 5 hours the charcoal was collected on hardened, ash-free filter-paper (Schleicher and Schüll, No 575), being transferred completely by means of the buffered filtrate. The charcoal was washed with 3·0 cc. of distilled water, and dried on the filter-paper over phosphoric oxide for 15–18 hours. The charcoal and filter-paper were then eluted with 5·0 cc. of 98–100 % acetic acid for 24 hours at room temperature, under which conditions the oxytocic substance is stable [Kamm *et al.*, 1928]. It is highly probable that the duration of this stage might be considerably curtailed. The charcoal was removed by filtration, washed with 5·0 cc. of 98 % acetic acid and then with 3·0 cc. of distilled water, and the combined filtrate and washings were evaporated to dryness at 40° under reduced pressure. The clear residual gum was dissolved in water, filtered through ash-free paper, and made up to 10·0 cc. at p_H 3. After some experience it was possible to judge exactly the point at which to interrupt the evaporation under reduced pressure, so that the aqueous solution should be at p_H 3, but if necessary the adjustment was made by the careful addition of acetic acid. No sodium hydroxide or acetate was added.

Estimation of solids. 6·0 cc. of the solution (above) were evaporated to constant weight in a tared basin at 100°, and the weight of solid which had not been adsorbed from 1 cc. of the stock solution was calculated from the weight of the residue and the initial content of solid.

The remaining 4 cc. were sealed up in sterile ampoules and preserved at 0° for the oxytocic assay.

Controls. A control experiment was carried out simultaneously with each adsorption in order to ascertain that the oxytocic activity suffered no destruction at the p_H of the experiment. 1·00 cc. of the stock solution was brought to the required p_H, mixed with 3·0 cc. of the requisite buffer solution cooled to 0°, and the mixture placed in the refrigerator at 0° side by side with the adsorption

experiment. After 5 hours, the control solution was made up to 10 cc. at p_H 3, sealed up in a sterile ampoule, and preserved for testing.

The oxytocic assay. The oxytocic activity of each sample was assayed by the degree of contraction it produced in the isolated guinea-pig's uterus, the method followed being that of Dale and Laidlaw [1912] as modified by Burn and Dale [1922] and Burn [1928]. The Ringer solution used was the modification recommended by Burn. Virgin guinea-pigs ranging from 180 to 250 g. in weight were employed; those weighing less than 200 g. were found to be most suitable. The original Kamm solution was used as a standard in the actual tests, but, for convenience, the results are expressed in International Standard Units, the Kamm solution having been assayed at 200 units per cc. The possibility of deterioration of this standard was reduced to a minimum by keeping it at 0° in half a dozen sealed ampoules, each containing about 10 cc. of a 1 : 20 dilution (0·50 cc. made up to 10·0 cc. at p_H 3) of the Kamm solution; the activity of each ampoule was checked against the one previously used. It should be noted that standard solutions prepared from the stock Kamm solution on January 12th, February 27th and July 12th, had the same oxytocic activity; this fact will be referred to later.

A typical test is reproduced in Fig. 1. A uterus frequently exhibits a spontaneous alternation of large and small contractions, and the method of

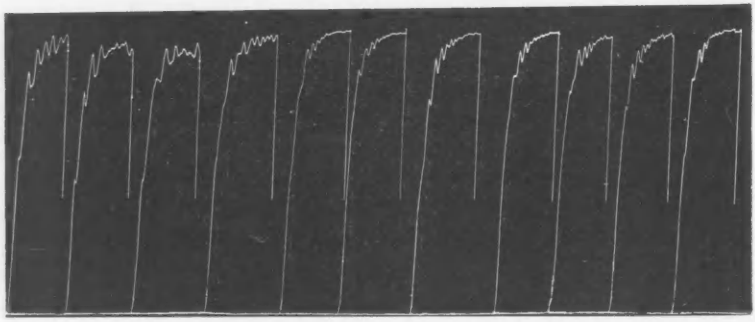

Fig. 1. Method of performing the oxytocic assay: standardisation of solution IV in terms of solution B (original Kamm solution).

The tracings are records of the contractile responses of the same horn of a guinea-pig's uterus (suspended in 100 cc. of modified Ringer solution) to successive doses of appropriate dilutions of solutions IV and B. Beneath each tracing are noted the dilution, dose and time (in minutes after the hour) at which the dose was given. The comparison between the oxytocic activities of two doses is made by measuring the maximum heights attained in the responses; the dose consistently giving the greater contraction has the higher oxytocic activity.

grouping the contractions as shown is designed to obviate error from this source[1]. No systematic attempt was made to arrive at absolutely equal contractions of the uterus when standard and unknown were administered, but the least dose of standard clearly greater than the unknown and the greatest dose of standard clearly less than the unknown were ascertained. The activity of a sample was taken as the mean of these two values, which are recorded in Table I, and are represented in Figs. 3 and 4 by the upper and lower limits

Table I. *Kamm solution.*

Initial solid 2·5 mg. per cc. Initial activity 200 units per cc.

Exp.	Age in days of Kamm soln.	p_H	Oxytocic activity recovered from adsorption Maximum: 200 Inter. units Limits		Mean	Oxytocic activity controls Maximum: 200 Inter. units Limits		Mean	Solid in 6 cc. soln. obtained from adsorption exp. mg.	Solid not adsorbed per cc. of Kamm soln. mg.
I	1	3·8	127	100	114	—	—	195	1·5	1·2
II	1	4·8	160	120	140	250	170	210	2·0	0·8
III	1	5·7	75	50	63	197	167	182	1·8	1·0
IV	9	7·0	100	80	90	250	160	205	3·2*	−0·1‡
V	9	9·0	175	120	148	250	165	208	2·7	0·2
VI	9	11·0	200	125	163	250	160	205	2·4	0·5
VII	17	13·0†	250	75	163	266	150	208	1·8	1·0
VIII	17	1·0†	165	100	133	—	—	—	2·0	0·9
IX	24	2·9	200	100	150	—	—	—	1·3	1·4
X	24	2·0†	230	165	198	—	—	—	1·4	1·3
III A	28	5·7	134	100	117	250	160	205	1·7	1·1
IV A	28	7·0	200	150	175	250	160	205	1·8*	1·1‡
IV B	32	7·0	200	150	175	—	—	—	1·5*	1·3‡
II A	32	4·8	187	135	161	—	—	—	1·4	1·3
VI A	35	11·0	250	160	205	250	160	205	1·1	1·6
VII A	35	13·0†	—	—	—	—	—	—	1·7	1·1
IX A	39	2·9	190	150	170	—	—	—	1·4	1·3
V A	178	9·0	—	—	—	—	—	—	1·5	1·2

* In Exp. IV part of the residue obtained by evaporating the acetic acid elution under reduced pressure dissolved readily in water, and left a white caseous precipitate which only passed slowly into solution. This sequence was not observed in the corresponding experiments IV A and IV B.

† The p_H for these estimations was adjusted by bringing the sample of Kamm solution to the required value as closely as possible and adding 6 cc. of 0·1 *N* sodium hydroxide, 0·1 *N* or 0·01 *N* hydrochloric acid as required.

‡ Corrected for weight of phosphate buffer adsorbed, see p. 346.

respectively of vertical lines, whose mid-points represent the mean values. A difference of not more than 20 % between mean and extremes was aimed at, but not attained in every case. The extremes were, however, always demonstrated at least as clearly as in the example given (Fig. 1), and the actual error of any determination must lie well within the limits of the vertical line by which it is represented in Figs. 3 and 4. This is confirmed by two observations, *viz.* (*a*) the constant nature of the value obtained for the control solutions, whose theoretical total strength was 200 units[2]: and (*b*) the clear demonstration of difference in strength between samples II and II A (Table I), for which the vertical lines representing limits overlap; this difference corresponds

[1] We are indebted to Dr J. H. Burn for the opportunity of seeing standardisation carried out in this way

[2] Three times the standard deviation of the individual values from the true value of 200 is 26, *i.e.* an error of ±13 %.

almost exactly with the previously obtained difference between their mean values. Unfortunately, these were the only two samples with overlapping ranges of value which were compared directly with each other as well as individually against the standard; the value of such supplementary determinations was not realised, since it had been arranged that the previous history of the samples was unknown during the testing.

Discussion of results of experiments.

The results are recorded in Table I.

Since the standard solutions were assayed in International Units, it was possible to determine the maximal total activities which might be recovered from the adsorptions or contained in the control solutions, and these values are noted at the head of their respective columns.

In order to maintain uniformity in the table, the figures in the column marked "Oxytocic activity recovered from adsorption" have been amended so as to refer to 1 cc. of stock Kamm solution, although the volume actually used for the adsorption experiments was 2 cc.

From the column marked "Oxytocic activity of controls" it will be seen that the oxytocic substance in a Kamm solution is stable for 5 hours at 0° between p_H 1 and 13. This result is in agreement with the observations of Gaddum [1930] who used whole extracts of the posterior lobe.

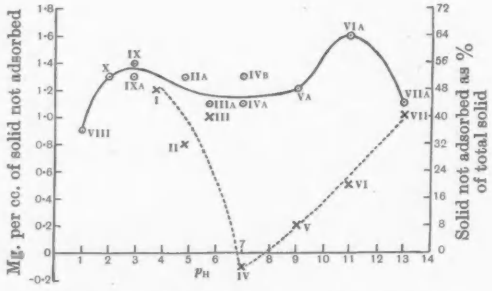

Fig. 2. Solid not adsorbed plotted against p_H. Initial solid 2·5 mg. per cc.

The results are also expressed graphically. Fig. 2 represents the effect of hydrogen ion concentration on the weight of solid which was not adsorbed from each cc. of the Kamm solution, and this weight is also expressed as a percentage of the initial content. Fig. 3 shows the influence of hydrogen ion concentration on the amount of activity recovered from the charcoal. It will be seen that in both figures the points lie on two curves. In each case, the dotted curve represents adsorptions I–VII carried out during the first 17 days after the preparation of the Kamm solution. The full curves refer to adsorptions

made during the following three weeks, the sole exception being that marked VA, which is of later date.

In general, the differences between the dotted and full curves, as regards either solid not adsorbed or activity recovered, lie outside the limits of experimental error. This fact suggests that the contents of the stock Kamm solution have undergone some radical change even when kept at 0°, and a consideration of the dates of the experiments points to the occurrence of this change before 3 weeks have elapsed from the date of preparation of the solution. It is possible that this change occurs earlier, but information on th is point cannot be obtained from this series of experiments.

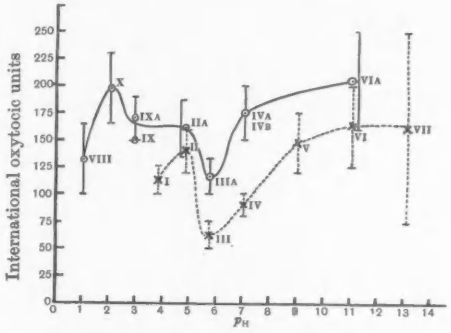

Fig. 3. Activity recovered, per cc. of original Kamm solution, plotted against p_H.

The adsorption VIA at p_H 11 removed 64 % of the solid from the Kamm solution, but it should be noted that adsorptions at the same p_H made 5 and 7 months after VIA failed to remove more than 40–48 % of the solid. These later values (not shown in Fig. 2) thus fell roughly on the straight line which indicates 42–50 % non-adsorption of solid, and which represents approximately the general behaviour of the more mature Kamm solution when submitted to adsorption between p_H 2 and 13. Adsorption VIA is therefore unique in this series, but since the maximal activity was recovered in that adsorption, it is improbable that the high value for "solid not adsorbed" is due to loss during the experiment. It is possible, however, that slight variations in hydrogen ion concentration might greatly affect the percentage of solid not adsorbed at any given p_H, and we intend to make a closer study of adsorptions at several points on the p_H scale. An alternative explanation is that with the lapse of time the adsorbability of part of the contents of the Kamm solution became modified. Our experiments indicate that such modification may take place shortly after the preparation of the solution, and the occurrence of a change of this nature at a later date must therefore be considered. Whether the oxytocic substance is in chemical or physical combination with non-oxytocic material,

or whether there is no direct connection, the apparently smaller adsorption of oxytocic activity from the fresh stock solution as compared with that from the same solution at a later date could be the result of preferential adsorption of inactive molecules at the expense of active ones, or of actual diminution of the oxytocic effect by an inhibitory substance whose adsorbability subsequently became reduced, or of modification of the molecules of the oxytocic substance itself so that their adsorbability was increased with lapse of time. Whatever the explanation may be, the difference observed cannot be due to increase in the activity of the oxytocic substance itself, since the standard solutions, prepared at intervals of 6 weeks and 6 months show the same oxytocic activity (p. 342); one standard solution was prepared at the same time that the first adsorption was carried out.

Solid contributed to charcoal by buffer salts. The amount of solid contributed to the charcoal by the buffer solutions has been shown to be negligible except at p_H 7. The procedure has been to repeat the adsorptions on a scale ten times as great as that of the actual experiments, replacing the oxytocic solution by distilled water. The corrections for the amount of solid contributed by the buffer are therefore one-tenth of the weights recovered from the charcoal, and are recorded in Table II. These corrections have been neglected, except in the case of experiments at p_H 7.

Table II.

Exp.	Solid recovered from charcoal (mg.)	Correction (mg.)
Control*	1·1	—
p_H 3·8	0·8	−0·03
p_H 4·8	1·0	−0·01
p_H 5·7	0·9	−0·02
p_H 7	2·4	0·13
p_H 9	1·3	0·02
p_H 11	1·0	−0·01

* Represents solids contributed by charcoal, distilled water and acetic acid.

Ratio of activity/solid.

Fig. 4 shows the variation of the ratio activity/solid with change in p_H of the adsorption. The ordinates represent the activity in international oxytocic units per mg. of solid, the abscissae the p_H. The activity of the solid from which solution VIA (p_H 11) was made was thus 225 units per mg., whereas the posterior lobe powder, when fresh, had an activity of 1·5 units per mg., and the Kamm stock solution an activity of 80 units per mg. The contraction used in making the tests of VIA was given by a dilution of 1 : 550,000,000; this contraction was not quite maximal.

Non-adsorbed oxytocic substance. Oxytocic assays have not been made of the non-adsorbed active substance left in the filtrates from the charcoal adsorptions. The control experiments, however, indicate that this activity has not been destroyed, and it will be observed that at p_H 7 (Exp. IV) the fresh

Kamm solution apparently yielded all its glandular material to the charcoal, whereas only 45 % of the activity was adsorbed. It would thus appear that the non-adsorbed oxytocic substance must be associated with a very minute amount of glandular material in the filtrate. Experiments are in progress to utilise this fact in the purification of the oxytocic substance.

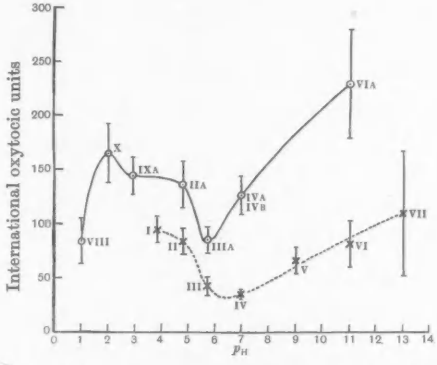

Fig. 4. Activity per mg. of solid adsorbed plotted against p_H.*

Summary.

1. Norite charcoal adsorbs oxytocic activity from a partially purified extract of the posterior lobe of the pituitary gland.

2. The adsorption is continuous between p_H 1 and 13, and the active substance may be eluted by glacial acetic acid.

3. The oxytocic activity which was recovered in adsorption experiments with a freshly prepared, partially purified extract varied from 50 to 75 % of that of the same solution 3 weeks later, even when this was kept at p_H 3 and 0°. In the case of the more mature solution, the amount of activity recoverable was about 80 % of the total, taking an average over the range p_H 1 to 13. It fell to a minimum of 55–60 % at p_H 5·7, but at p_H 2 and p_H 11 rose to 100 % within the limits of error of the method of assay.

4. There was no marked difference in the shapes of the curves representing the influence of hydrogen ion concentration on the amount of active substance adsorbed from fresh or more mature solutions.

5. Using the fresh, partially purified extract the amount of solid not adsorbed was about 50 % of the total at p_H 3·8 and 40 % at p_H 13. Between these limits the weight of solid not adsorbed fell, and was zero at p_H 7.

6. Using the more mature extract, the amount of solid not adsorbed was about 50 % of the total present, taking an average between the limits p_H 1 and 13. This value rose to 64 % at p_H 11, but adsorptions at p_H 11 made several months later failed to remove more than 40–50 % of the solid material.

7. The most active preparation contained 225 international oxytocic units per mg., and gave a not quite maximal contraction of the isolated uterus of the virgin guinea-pig at a dilution of 1 : 550 millions.

In connection with the oxytocic assay, our best thanks are due to Prof. Lovatt Evans for his assistance, and to Dr J. H. Gaddum for advice.

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XXXIX. THE ABSENCE OF ABNORMAL FORMS OF GLUCOSE FROM BLOOD DIALYSATES.

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Comparisons of the optical activity and copper-reduction values of blood filtrates and dialysates have led certain observers to conclude that these aqueous solutions contain γ-glucose or other labile form of glucose [Winter and Smith, 1923; Lundsgaard and Holbøll, 1924, 1925; and in particular, Thomas, 1931]. Misapprehension of the behaviour of γ-glucose had led these workers to attribute to it the undoubted fact that the optical activities of blood dialysates are lower than the theoretical values deduced from the copper-reducing powers of the solutions.

Although numerous comparatively stable derivatives of γ-glucose (glucofuranose) are known, the free compound is unknown, and in aqueous solution instantly reverts to the ordinary equilibrated mixture of the stable α and β forms, showing $[\alpha]_D + 52{\cdot}5°$. (It is possible that a certain amount of γ-sugar may always be present in the equilibrium mixture but this would not then constitute an abnormality.)

To what then, can we attribute the discrepancies between reducing power and optical activity? Diminution of the rotatory power of glucose solutions has been shown to be caused by the presence of amino-acids and urea [Waterman, 1917, 1, 2, 1918; Euler *et al.*, 1926; Denis and Hume, 1924; Hynd, 1926, *etc.*]. While the concentrations within the blood of these substances are admittedly low, it should not be overlooked that their influence may have a slight effect in causing the differences noted.

Visscher [1926] was the first to attribute the phenomenon to the laevorotatory effect of salts of *d*-lactic acid, already shown by von Noorden [1912] to be formed from glucose by the glycolytic action of blood. In support of this view he called attention to the fact that the optical activity of blood filtrates altered with change in p_H. A similar effect was observed by Anderson and Carruthers [1926]. Wright, Herr and Paul [1928] succeeded in isolating zinc *d*-lactate from glycolysed blood, thus providing strong evidence in support of Visscher's theory.

In the recent paper by Thomas [1931] the work on *d*-lactic acid appears to have been disregarded, for the γ-glucose theory is again put forward. It was therefore considered expedient to endeavour to correlate the observed optical

activities of blood dialysates with the theoretical values calculated from both the sugar and the lactic acid contents. To this end fresh blood was obtained from cats, rabbits and sheep, some normal, others with high blood-sugar levels, induced by decerebration or by adrenaline injection, whereby it was possible to obtain dialysates with widely varying concentrations of glucose and of lactic acid.

The results of the experiments showed that the ideas of Visscher could be fully substantiated, as the amounts of lactic acid found in the dialysates were sufficient to account for the rotation discrepancies to well within the bounds of observational error. The polarimeter employed permitted the use of a 4 dm. tube and an accurate reading of 0·035° could be made. While errors of observation below 0·10° are unavoidably high, it will be seen that, in these experiments where the readings were greater than 0·035°, the mean error between observed and calculated activities was 0·020°.

Experimental.

Determination of specific rotation of sodium d-*lactate.*

Purdie and Walker [1893] quote $[\alpha]_D - 12{\cdot}15°$ for $c = 0{\cdot}075N$.

Concentration of sodium *d*-lactate %	$\alpha^{16°}$	$[\alpha]_D^{16°}$
0·8752	−0·415°	−12·86°
0·3501	−0·208	−14·85
0·1403	−0·087	−15·50

For the following calculations sodium *d*-lactate was assumed to display the approximate $[\alpha]_D^{16°} = -16{\cdot}0°$.

Dialysis of the blood and examination of the dialysates.

30 cc. of freshly drawn blood were dialysed in 10 portions of 3 cc. each into equal volumes of normal saline at 16°. After 30 minutes the clear dialysate was examined polarimetrically in a 4 dm. tube at 16°, the zero being determined immediately afterwards, with the same tube filled with distilled water. A portion of the dialysate was employed for the sugar estimation by a modified Shaffer-Hartman method, and the remainder, after removal of the sugar by the copper-lime precipitation method, was employed in the determination of the lactic acid by the procedure of Friedemann, Cotonio and Shaffer [1927].

Sources of blood employed.

Sheep (I to V) all normal.

Rabbits (VI to X). Normal (XI). Injected adrenaline, then amytal. Blood-sugar 334 mg./100 cc.

Cats (XII to XIV). Decerebrated (XV). Decerebrated and eviscerated.

In the experiments of Table I the amounts of sodium *d*-lactate were determined as lactic acid.

Table I. *Analytical data of dialysis experiments.*

Experiment	Sugar mg./100 cc.	Sodium *d*-lactate mg./100 cc.	Observed α °	α calculated from sugar °
I (Sheep)	18·4	15·4	+0·00	+0·039
II ,,	14·8	12·9	+0·035	0·031
III ,,	16·0	6·9	+0·00	0·034
IV ,,	16·8	nil	+0·035	0·035
V ,,	20·0	23·7	+0·028	0·042
VI (Rabbit)	38·4	87·3	+0·070	0·080
VII ,,	37·6	46·9	+0·070	0·079
VIII ,,	28·0	51·7	+0·035	0·059
IX ,,	28·0	41·8	+0·035	0·059
X ,,	24·8	70·2	+0·00	0·052
XI ,,	116·0	56·1	+0·208	0·254
XII (Cat)	164·0	38·0	+0·311	0·350
XIII ,,	78·4	51·1	+0·105	0·187
XIV ,,	88·8	103·4	+0·120	0·165
XV* ,,	47·2	117·5	−0·035	0·099

* The dialysis in this experiment was imperfect, as the dialysate had a faint pink tinge.

The values obtained from the sheep blood were too low to admit of polarimetric correlation; they are included however to demonstrate the presence of lactic acid in these dialysates.

Table II contains the polarimetric figures derived from those experiments in which the optical activity was greater than the minimum of discrimination (0·035°).

Table II.

α_G is rotation calculated for % glucose found.
α_L ,, ,, sodium *d*-lactate.

Experiment	α_G °	α_L °	$\alpha_G + \alpha_L$ °	Observed α °
XII	+0·346	−0·015	+0·331	+0·311
XI	+0·245	−0·022	+0·223	+0·208
XIV	+0·187	−0·039	+0·148	+0·105
XIII	+0·167	−0·019	+0·148	+0·120
XV*	+0·099	−0·045	+0·054	−0·035
IX	+0·059	−0·015	+0·044	+0·035
X	+0·052	−0·027	+0·025	+0·00
IV	+0·035	0·00	+0·035	+0·035

It will be seen that in all the observations (excepting experiment XV where dialysis was imperfect), a mean difference is disclosed of only 0·020° between the calculated and the observed optical activities.

Conclusion.

It is concluded that in all the specimens of blood examined, the apparent low optical activities of the dialysates may be accounted for by the presence of the laevorotatory sodium *d*-lactate, without assuming the presence of any γ-glucose.

The writer wishes to thank Prof. J. J. R. Macleod, for suggesting this problem, and for the constant interest which he took in the work.

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XL. AN IODIMETRIC METHOD FOR THE DETERMINATION OF IRON IN BLOOD.

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In the course of certain investigations, at present being carried out at this Institute, it became necessary to employ a routine but accurate method for the estimation of iron in small quantities of blood.

The well-known reaction of ferric ions with thiocyanate is the basis of colorimetric procedures used by Wong [1923], Walker [1925] and others, but the disadvantages attending the colorimetric comparison inclined us against the adoption of these methods.

Jahn [1911] has described a procedure in which the blood is submitted to dry ashing and the iron residue titrated with titanous chloride in the presence of potassium thiocyanate. We have found dry ashing of the blood to be unsatisfactory on account of the difficulty of obtaining the iron residue in a form in which it is completely soluble in concentrated hydrochloric acid. Moreover it is necessary to carry out titanous chloride titrations in an atmosphere of carbon dioxide.

Murray [1924] has employed dry ashing of the blood, followed by reduction of the iron, which is then titrated with standard permanganate in an atmosphere of hydrogen. Using dilutions commensurate with the small quantities of iron present in blood the permanganate end-point is difficult to judge and is therefore unreliable.

Accordingly we have developed an iodimetric method which, employing the sensitive starch iodide indicator, gives results accurate to about 2 % on 2 cc. portions of blood.

The reaction between ferric and iodide ions proceeds according to the reversible equation

$$Fe^{+++} + 3I^- \rightleftharpoons Fe^{++} + 2I^- + \tfrac{1}{2}I_2.$$

We have been unable to find any information on the state of equilibrium of this system at the dilutions which must be employed in the analysis of small quantities of blood. Our first efforts were accordingly directed to finding conditions suitable for the employment of the above reaction in the estimation of the amount of iron in 2 cc. of blood (about 0·7 mg.).

It was found that in neutral solution the equilibrium of the iron-iodide

system lies only a short distance from the left and is therefore useless for quantitative purposes. With increasing hydrogen ion concentration, however, the equilibrium moves towards the right, until in sufficiently acid solution quantitative reduction takes place. The liberated iodine may then be removed by titration with $N/200$ thiosulphate solution.

For our present purpose it will be sufficient to refer to Table I which summarises the results of a series of titrations carried out by us. The figures were obtained by titrating with $N/200$ thiosulphate the iodine liberated by the interaction of a known quantity of ferric iron with potassium iodide.

Table I.

Initial concentration of iodide, 50 mM per litre.
Concentration of hydrochloric acid, 5 M.

Initial concentration of ferric salt (mM)		1	2	3	4	5	6	7	8	9	10
Thiosulphate titration of 10 cc. of solution	Found at equilibrium	1·97	3·97	2·96	3·94	2·505	2·96	3·48	3·99	4·45	4·95
	Calculated for complete reduction	2·00	4·00	3·00	4·00	2·50	3·00	3·50	4·00	4·50	5·00
Normality of thiosulphate		0·005	0·005	0·01	0·01	0·02	0·02	0·02	0·02	0·02	0·02
Ferrous salt at equilibrium (%)		98·5	99·2	98·6	98·5	100·2	98·6	99·4	99·7	98·8	99·0

It will be seen that in solutions which are 5 M with respect to HCl and 50 mM with respect to potassium iodide it is possible to estimate quantitatively amounts of iron representing concentrations of from 1–5 millimols per litre. Under these conditions the end-point is sharp and there is no return of colour within 10 minutes.

It remains therefore to develop a preliminary treatment of 2 cc. of blood which will allow of the above conditions in the final titration. In practice it was found that the following method gave satisfactory results.

Method.

The following reagents, which should be free from iron, are required.

Sulphuric, nitric and hydrochloric acids.

Potassium iodide (4 % solution—free from iodate: this is made up fresh each day).

Starch indicator (1 % solution of starch in sat. sodium chloride).

$N/200$ sodium thiosulphate.

Merck's perhydrol.

Potassium dichromate.

Preparation of thiosulphate. $N/200$ sodium thiosulphate is best prepared according to the instructions given by Pregl [1930]. $N/10$ sodium thiosulphate, which has been ripened to constant strength, is diluted exactly to $N/200$ strength by means of boiled out distilled water. The diluted solution contains 5 % by volume of amyl alcohol as a preservative and we have found that, when kept in amber-coloured bottles attached to automatic burettes protected by soda-lime tubes, the reagent retains its strength over long periods.

Standardisation of thiosulphate. 5 cc. of conc. hydrochloric acid are placed in an $8 \times 1\frac{1}{4}$ in. pyrex test-tube, and boiled gently for 1 minute, a glass bead being added to prevent bumping. The tube is then cooled under the tap and 3 cc. of standard potassium dichromate solution (25 mg. $K_2Cr_2O_7$ in 100 cc.) and 2 cc. of 4 % potassium iodide solution are added. A tightly fitting rubber stopper is then inserted and the tube is allowed to stand for exactly 10 minutes. The thiosulphate solution is then added drop by drop with shaking until the yellow colour of the solution almost disappears. At this point 10 cc. of distilled water are added followed by 5 drops of the starch indicator when the characteristic blue colour of the starch iodide complex develops. After shaking, thiosulphate is added until the blue colour disappears. The last few drops should be added very slowly, drop by drop, and with constant shaking.

A blank titration is carried out exactly as above except that 3 cc. of distilled water take the place of the dichromate solution. The value of the blank is subtracted from that of the first thiosulphate titration.

Normality of thiosulphate $= \frac{3x}{4{\cdot}9y}$.

Where $x =$ g. $K_2Cr_2O_7$ in 100 cc. of solution and $y =$ cc. of thiosulphate required.

The estimation of iron in blood.

2 cc. of blood are drained under the surface of 2 cc. of distilled water contained in a pyrex test-tube, the pipette (which is graduated to contain and not to deliver) being rinsed twice with the supernatant water.

5 cc. of conc. HNO_3 are then added and the tube is placed in a bath of boiling water until only 2 cc. of pale yellow solution are left. In this way the blood-proteins are digested smoothly and most of the organic matter is oxidised.

The latter part of the combustion is best carried out by hand. 0·5 cc. of conc. H_2SO_4 is added to the tube along with a glass bead to prevent bumping and the liquid is boiled gently over the flame of a small burner until charring is complete. The tube is allowed to cool a little, 1 cc. of conc. HNO_3 is added and the liquid is again boiled. If any organic matter still remains a few more drops of HNO_3 are usually sufficient to complete the destruction. 1 cc. of distilled water and 5 drops of perhydrol are added to the cooled liquid which is again boiled until white fumes appear. Two successive quantities of 1 cc. of distilled water are now added and after each addition the liquid is evaporated down in order to remove any remaining HNO_3 or perhydrol.

5 cc. of conc. HCl are introduced into the cooled tube and the liquid is boiled gently until a clear yellow solution is obtained. The tube is cooled under the tap, 3 cc. of distilled water and 2 cc. of 4 % KI solution are added immediately and the tube is stoppered tightly.

The titration is then carried out, as before, at the end of exactly 10 minutes.

A blank estimation is carried out using 2 cc. of water instead of blood. The blank value is subtracted from the titration figure. In practice we have found

that the blank value remains constant and need only be redetermined when any change is made in the reagents.

Mg. iron per 100 cc. blood = 2792 xy.

Where x = cc. of thiosulphate required and y = normality factor of thiosulphate.

Results.

The following table gives the results of estimations in which a solution of ferrous iron of known strength was used instead of blood. The complete procedure outlined above was followed.

Table II.

mg. Fe present	mg. Fe found	Percentage error
0·6055	0·6149	1·4
0·8074	0·8088	0·17
1·0093	0·9954	−1·3
1·2111	1·1920	−1·6

The following experiment, using cow's blood, illustrates the recovery of iron added to the blood before ashing.

Table III.

Fe in 2 cc. of blood mg.	Fe added mg.	Total Fe mg.	Fe found mg.	Fe recovered %
0·6829	—	0·6829	0·6829	—
0·6829	0·2830	0·9659	0·9576	99·2
0·6829	0·4000	1·0829	1·067	98·5
0·6829	0·4800	1·1629	1·143	98·2
0·6829	0·5600	1·2429	1·214	97·8

We have now carried out analyses on over 50 samples of cow's blood and have obtained excellent agreement between duplicates.

Summary.

An iodimetric method is described for the estimation of iron in 2 cc. of blood.

Our thanks are due to Dr N. C. Wright for his interest in this work and to Mr L. Jordan who obtained blood samples for us.

One of us (J. H. B.) is indebted to the Trustees of the Carnegie Research Fund for a Scholarship during the tenure of which this work was carried out.

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XLI. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

I. THE KAUFMANN-MAGNE TECHNIQUE.

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The object of the present series of papers is to effect a correlation of our knowledge of milk secretion and of the chemistry of the blood.

Since the process of milk secretion involves complex molecular transformation within the secreting cells of the mammary gland, it is a matter of physiological importance to determine which constituents of the blood are selected by the gland for conversion into milk protein, carbohydrate and fat. Such data are also of immediate practical importance since effective control of the milk production of animals requires a co-ordination of diet and milk yield and, therefore, an adequate knowledge of the relationship between active gland and blood-stream—the intermediary between gland and digestive tract.

The secretory process of the mammary gland is extremely sensitive and can best be studied in the intact animal. The ideal method, therefore, of determining whether any given blood constituent takes part in the secretory process is to compare the concentration of that constituent in the blood before entering and after leaving the gland. A diminished concentration in the efferent blood would indicate utilisation by the mammary gland.

The mammary glands of the cow are drained by the abdominal subcutaneous veins which are superficial and therefore readily accessible without disturbance to the animal. The arterial blood, however, is supplied by the mammary artery which is deeply embedded in the tissues and is inaccessible without serious disturbance to the animal.

The problem of securing samples of arterial blood with which the comparison of analytical figures for mammary venous blood is justified is therefore one of considerable practical difficulty and was first encountered by Kaufmann and Magne [1906] who wished to investigate the consumption of blood-glucose in the gland under varying conditions of activity.

These workers chose the concentration of sugar in the blood of the jugular vein as a standard with which to compare the concentration of sugar in the mammary venous blood. In this assumption that jugular blood may be taken

to represent mammary arterial blood they have been followed by Meigs, Blatherwick and Cary [1919] and Cary [1920], the former in a study of the phosphorus metabolism of the mammary gland, and the latter in support of a claim that blood-amino-acids are the precursors of milk-protein. The intervention of tissue metabolism between arterial blood and jugular (venous) blood renders analytical values for the latter difficult of interpretation, an aspect which is to be considered in later papers.

In addition, there are other factors, unrecognised by previous workers, which may effect modifications in the venous blood draining from either mammary tissue or from the tissues of the head and neck. Before proceeding to the comparison of the concentrations of any given constituent in two bloods drawn from different sources, it must be ascertained that no water has been lost from the blood either to the tissues or outside the body. The lactating cow on the average secretes over a period of 12 hours about 10 litres of water as milk. This must result in concentration of the blood and we have to consider whether the haemo-concentration so produced is of sufficient magnitude to be reflected in analytical figures.

A secretion of 10 litres per 12 hours is equal to a secretion of about 14 cc. of water per minute and would therefore require a velocity of flow of blood through the mammary gland of only about 1·5 litres per minute to give a 1 % concentration of the blood. As the velocity of flow of blood is probably very much higher than this it follows that the haemo-concentration due to secretion of water by the mammary gland is negligible, so far as analytical figures obtained for mammary venous blood are concerned.

The loss of water from the blood in its passage through the tissues of the head and neck might, however, be a quantity of greater magnitude. Schalk and Amadon [1928] estimate the activity of the salivary glands of the ox as constant at some 60 or more litres of saliva per 24 hours. Such a secretion may make heavy demands on the water of the blood supply, and when one considers that part of the blood in the jugular vein is drained from the salivary glands, it is apparent that this constant flow of saliva may result in appreciable haemo-concentration in the jugular venous blood.

Although previous workers have failed to recognise that analytical values for jugular and mammary bloods may not be strictly comparable there are indications of such a possibility to be found in their work. For example, Meigs *et al.* [1919] have noted that samples of jugular and mammary bloods taken simultaneously from the same animal do not always have the same percentage corpuscular volumes. They do not discuss this fact fully in their paper nor do they seem to have realised the possible effect of such a difference on their analytical values for other blood constituents.

Accordingly the first question we have investigated in this experimental survey is that of the concentrations of indiffusible constituents in samples of blood taken simultaneously from different regions of the bodies of lactating and non-lactating animals.

The constituents chosen—percentage corpuscular volume and iron content—are those most likely to be accurate indices of the amounts of water contained in equal volumes of blood from different sources.

Experimental.

Samples of blood have been taken simultaneously from the radial artery and the jugular and abdominal subcutaneous (mammary) veins of lactating and non-lactating cows.

The samples, each of about 60 cc. volume, were drawn into dry sterile glass bottles containing 0·1 g. sodium oxalate as anticoagulant. They were immediately examined.

1. *Corpuscular volume* was estimated by centrifuging in haematocrit tubes until constant volume was attained. As each blood contained the same amount of anticoagulant the slight effect of the addition of sodium oxalate on corpuscular volume is eliminated.

2. *Iron content* was estimated by a micro-method elaborated for the purpose of this investigation (see previous paper).

(*Note.* We have found arterial puncture of non-aesthetised cows to be a procedure attended by considerable disturbance with most animals. Only in eight cases have we obtained full blood samples from all three sources without disturbance.)

The results of analyses of samples of bloods from 17 different animals are given in Table I.

Table I.

Cow No.	Lactating or non-lactating	Corpuscular volume (%)			Iron content (mg. Fe per 100 cc. blood)		
		Jugular	Arterial	Mammary	Jugular	Arterial	Mammary
1	Lactating	33·5	30·5	30·5	37·04	35·26	35·06
2	,,	36·5	34·0	33·5	42·32	39·34	38·94
3	,,	41·0	39·0	39·0	38·75	37·71	37·33
4	,,	39·0	38·0	37·0	36·69	35·14	34·26
5	,,	37·0	36·0	36·0	40·86	39·29	39·50
6	,,	36·0	—	34·5	39·65	—	38·39
7	,,	33·0	—	31·0	40·25	—	38·66
8	,,	36·0	—	36·0	37·96	—	37·07
9	,,	43·0	—	42·0	38·75	—	36·94
10	,,	42·5	—	40·5	41·83	—	39·77
11	Non-lactating	41·0	38·5	38·5	39·31	36·28	36·81
12	,,	36·5	35·5	35·5	34·74	—	32·94
13	,,	32·5	29·0	30·0	34·23	32·56	33·46
14	,,	38·3	34·3	34·8	37·28	34·60	34·89
15	,,	30·0	—	28·0	38·12	—	37·27
16	,,	34·0	—	31·5	43·56	—	41·88
17	,,	36·0	—	33·5	37·70	—	35·77

Table II has been constructed from the data of Table I and shows, in each case, the differences between jugular, arterial and mammary bloods expressed as percentages of jugular.

Table II.

Cow No.	Lactating or non-lactating	Differences in corpuscular volume: Jugular-mammary as % jugular	Jugular-arterial as % jugular	Mammary-arterial as % jugular	Differences in iron content: Jugular-mammary as % jugular	Jugular-arterial as % jugular	Mammary-arterial as % jugular
1	Lactating	8·95	8·95	0·0	5·34	4·81	0·54
2	,,	8·23	6·85	1·3	7·96	7·04	0·92
3	,,	4·87	4·87	0·0	3·68	2·71	0·95
4	,,	5·14	2·57	2·57	6·63	4·24	2·30
5	,,	2·70	2·70	0·0	3·30	3·80	0·52
6	,,	4·10	—	—	3·20	—	—
7	,,	6·06	—	—	3·91	—	—
8	,,	0·0	—	—	2·30	—	—
9	,,	2·30	—	—	4·60	—	—
10	,,	4·70	—	—	4·90	—	—
11	Non-lactating	6·09	6·09	0·0	6·37	7·70	−1·3
12	,,	2·75	2·75	0·0	5·18	—	—
13	,,	6·24	9·37	−3·1	2·26	4·87	−2·6
14	,,	9·14	10·44	−1·5	4·50	6·42	−0·8
15	,,	6·66	—	—	3·03	—	—
16	,,	7·35	—	—	3·86	—	—
17	,,	6·90	—	—	5·11	—	—

Discussion.

The differences in the contents of indiffusible constituents are found in lactating and non-lactating animals alike, and cannot therefore be the result of the state of activity of the mammary gland. This conclusion receives support from the observation that mammary venous and radial arterial bloods bear a close resemblance to one another and that jugular blood differs from each in approximately the same degree.

Any differences between arterial blood and mammary venous blood analyses are not consistent and are not of a magnitude which may be regarded as significant when the various analytical procedures are considered. A scrutiny of the differences between jugular and arterial, and jugular and mammary bloods shows that they are consistently in the same direction and of a magnitude outside the range of any experimental error.

We are therefore forced to the conclusion that the circumstance responsible for the differences obtained is conditioned by interchange between blood and tissues in the course of the circulation of the blood through the head and neck. Loss of water from the arterial blood would cause an increase in concentration of venous blood constituents. This appears to be the point which our figures illustrate and we believe that the withdrawal of water from the arterial blood by the salivary glands is of sufficient magnitude to bring about the appreciable concentration of the blood resulting in a consistently increased value for corpuscular volume and iron content of jugular (venous) blood.

The concentration of diffusible blood-solutes, as well as of those indiffusible, is affected by loss of water *via* the salivary glands, and erroneous interpretations of analytical figures are likely to arise unless this fact is realised.

The determination of the degree of haemo-concentration however enables the effect of this factor on the concentration of a diffusible blood constituent to be allowed for.

Let x = mg. Fe per 100 cc. jugular blood;

y = mg. Fe per 100 cc. mammary blood;

then degree of concentration of jugular blood $= \frac{x}{y}$.

Let p = mg. sugar (or amino-N, *etc.*) per 100 cc. jugular blood;

then $p\frac{y}{x}$ = mg. sugar per 100 cc. mammary blood if no loss of water to the salivary glands had taken place.

The application of such a factor enables analytical values for jugular blood to be compared directly with values for arterial and mammary venous bloods since it eliminates the effect of loss of water to the salivary glands.

For the calculation of the correction factor we have chosen the values given by iron determinations in preference to those given by corpuscular volume measurements because the estimation of iron is not attended by the inaccuracies inherent in haematocrit technique.

In addition to the factors considered in this paper there is the influence of the lymph drainage on the concentration of venous blood-solutes. Sampling the lymphatic circulation would be a matter of great difficulty and could not be considered when drawing up this experimental plan. It is highly probable, however, that its effect would be in the same direction as that of the salivary glands but it is unlikely that its magnitude would be appreciable.

Summary.

1. The technique of Kaufmann and Magne as applied to the problem of milk secretion is discussed.

2. Experimental figures are given which show that the influence of salivary secretion on the analytical data of jugular (venous) blood is considerable.

3. A method of compensating for the influence of salivary secretion on these analytical figures is given, which permits the technique of Kaufmann and Magne to be applied to all diffusible blood solutes.

Our thanks are due to Dr N. C. Wright for his help and criticism and to Mr L. Jordan who obtained the blood samples. One of us (J. H. B.) is indebted to the Trustees of the Carnegie Research Fund for a Research Scholarship during the tenure of which this work was carried out.

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XLII. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

II. THE RELATION OF BLOOD-SUGAR ABSORPTION TO LACTOSE SECRETION.

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THE theory that the sugar of the blood is the precursor of lactose rests on experimental evidence which has been subjected to much criticism.

Bert [1884] removed the mammary glands of young goats and allowed the animals to become pregnant. Immediately after parturition sugar appeared in the urine. This did not take place with animals from which the mammary glands had not been extirpated before pregnancy. Bert concluded that immediately after parturition the blood receives an additional supply of sugar from some organ—presumably the liver, that normally this sugar is absorbed by the mammary gland for the synthesis of lactose and that, in the absence of the normal glandular function, the excess sugar is excreted in the urine.

This work was confirmed by Porcher [1909] who showed that the *post partum* glycosuria in animals from which the mammary glands had been removed was accompanied by hyperglycaemia and that the urinary sugar was glucose and not lactose. Neither Bert nor Porcher, however, was able to confirm these findings when guinea-pigs were used as experimental subjects. Evidence of a negative character was also obtained by Marshall and Kirkness [1907] while Moore and Parker [1900–01], who repeated Bert's original work, could not obtain unequivocal results.

Foa [1907, 1912] carried out perfusion experiments on the excised mammary glands of sheep. The glands were maintained at body temperature in Ringer's solution. Perfusion with Ringer's solution alone resulted in the secretion of a fluid free from lactose while on the addition of blood to the perfusate lactose was secreted. On replacement of the blood by glucose solution a watery fluid containing lactose was secreted by the glands, a result not obtained when galactose was used. These experiments have been criticised on the grounds that, in Ringer's solution, the excised glands rapidly became oedematous, a

condition in which the cells might be permeable to substances which would not normally penetrate the cell membrane.

In view of the unsatisfactory nature of the work reviewed above, the demonstration of the absorption of blood-sugar by the active mammary gland of intact animals becomes of considerable value.

The only previously recorded work of this description is that of Kaufmann and Magne [1906] who published data intended to illustrate the utilisation of blood-sugar by the mammary gland in different states of activity. From four cows (one of which was not lactating) these workers took samples of blood simultaneously from the jugular and abdominal subcutaneous (mammary) veins. They found that in the lactating animals the mammary venous blood contained less sugar than the blood from the jugular vein, while in their one non-lactating animal the two bloods had the same sugar content. On the basis of the finding of Chauveau [1856] that venous blood normally contains less sugar than arterial blood, Kaufmann and Magne suggested that jugular blood-sugar might be taken to represent the difference between arterial blood-sugar and the glucose oxidised in the cells of the mammary gland to provide energy. The difference between jugular and mammary blood-sugar levels would therefore represent sugar absorbed by the mammary gland for non-oxidative purposes. Such reasoning involves the assumption that the energy requirements of the mammary gland are identical with those of the tissues of the head and neck—the tissues drained by the jugular vein. Later workers have interpreted the results of this investigation as confirmation of the view that blood-sugar is the precursor of lactose, although this point is not explicitly made by Kaufmann and Magne.

The accurate estimation of the amount of blood-sugar absorbed by the mammary gland should be of value not only in deciding the nature of the precursor of lactose but also in problems concerning the relationship of mammary secretion to the general metabolism of the animal. There are certain considerations which suggest that the results of Kaufmann and Magne do not give a true measure of the amount of sugar absorbed from the blood.

In the first place the assumption that the energy requirements of the mammary gland are identical with those of the tissues of the head and neck remains as yet unjustified. This point can indeed only be decided when sugar values for arterial blood samples are available for comparison with those of jugular and mammary blood samples taken simultaneously. Further, since the investigation by Kaufmann and Magne was reported, it has become evident, principally from the work of Somogyi [1927, 1928, 1929], that a considerable fraction of the material estimated as blood-sugar by the older methods of analysis consists of non-sugar reducing substances. It is apparent that absorption of such substances by the mammary gland would result in a low "sugar" value for mammary venous blood and would lead to erroneous estimates of the amount of sugar utilised by the gland.

In the present investigation we have studied the problem of the sugar

uptake of the mammary gland by the analysis of samples of blood taken simultaneously from the radial artery and the jugular and mammary veins. Analytical figures for arterial samples should afford some information on the comparative energy requirements of the tissues of the head and neck and of the mammary gland, while the application of the factor established in the first paper of this series [1932, 2] eliminates the effect of salivary secretion. The method of sugar estimation employed ensures that the data shall be independent of the possible absorption of non-sugar reducing substances by the mammary gland.

Experimental.

Samples of blood were taken simultaneously from the jugular and mammary veins and radial artery of lactating and non-lactating cows. The samples were taken at 9 a.m., that is, four hours after milking in the case of the lactating animals. The samples, each of about 60 cc. volume, were drawn into clean glass bottles containing 0·1 g. sodium oxalate as anticoagulant. The blood-sugar analyses were carried out immediately.

The method of blood-sugar analysis employed was that of Hagedorn and Jensen as applied to Folin-Wu blood filtrates by Hiller, Linder and Van Slyke [1925]. We have chosen this method advisedly, because the Folin-Wu filtrate has been shown by Somogyi [1927] to give minimal precipitation of non-sugar reducing substances and we wished to have information of the amounts of such substances present in our samples. The total reducing power of the Folin-Wu filtrate from the freshly drawn blood was first determined. A sample of the blood was then incubated at 37° for 24 hours and the reducing power of the Folin-Wu filtrate from the incubated sample determined. The second figure gives the amount of non-sugar reducing substances in terms of glucose and the difference between the two figures gives the true sugar content of the blood as glucose. We have satisfied ourselves that incubation at 37° for 24 hours removes added glucose quantitatively.

The iron content of the blood samples was determined by the iodimetric method previously described by us [Blackwood and Stirling, 1932, 1].

In all we have taken samples of blood from thirteen animals—eight lactating and five non-lactating. In four cases, three lactating and one non-lactating, arterial samples were not obtained. In Table I are given the iron contents and true sugar values for all the blood samples obtained—in each case the values for jugular sugar are corrected, as indicated in the introductory part of this paper. In Table II are given the values for non-sugar reducing substances for each sample of blood—the jugular value being in each case corrected for loss of water to the salivary glands. In Table III the differences in blood-sugar content shown in Table I are expressed for comparative purposes as percentages of the arterial blood-sugar where arterial samples were obtained, and of the jugular blood where only jugular and mammary samples were available for analysis.

Table I.

No. of cow	Lactating or non-lactating	Iron content (mg. Fe per 100 cc. blood)			True sugar (mg. glucose per 100 cc. blood)			
		Jugular	Arterial	Mammary	Jugular	Jugular (corrected)	Arterial	Mammary
1	Lactating	37·04	35·26	35·06	53	50	52	47
2	,,	42·32	39·34	38·95	26	24	30	25
3	,,	38·75	37·71	37·33	37	36	49	33
4	,,	40·86	39·29	39·50	46	44	48	40
5	,,	36·69	35·14	34·26	51	49	56	41
6	,,	41·83	—	39·77	36	34	—	29
7	,,	37·96	—	37·07	43	42	—	40
8	,,	38·75	—	36·94	43	41	—	27
9	Non-lactating	39·31	36·28	36·81	44	41	46	44·5
10	,,	34·74	—	32·94	42	40	51	43
11	,,	34·23	32·56	33·46	49	47	56	51
12	,,	37·28	34·60	34·89	60	55	60·5	60·5
13	,,	37·70	—	35·77	43	41	—	44

Table II.

No. of cow	Lactating or non-lactating	Non-sugar reducing substances (as mg. glucose per 100 cc. blood)			
		Arterial	Jugular	Jugular (corrected)	Mammary
1	Lactating	23	26	25	27
2	,,	30	36	33	28
3	,,	30	31	30	32
4	,,	35	38	36·5	36
5	,,	28	33	32	25
6	,,	—	27	26	25
7	,,	—	33	32	27
8	,,	—	44	42	41
9	Non-lactating	28	28	26	29·5
10	,,	32	32	30	31
11	,,	27	20	19	22
12	,,	31·5	31	29	31·5
13	,,	—	40	38	37

Table III.

No. of cow	Lactating or non-lactating	Difference in sugar content of jugular, arterial and mammary bloods (as percentage arterial)		
		Arterial-jugular	Arterial-mammary	Jugular-mammary
1	Lactating	3·8	9·6	5·8
2	,,	20·0	16·6	−3·3
3	,,	26·5	32·6	6·1
4	,,	8·3	16·6	8·3
5	,,	12·5	26·7	14·2
6	,,	—	—	14·7*
7	,,	—	—	4·7*
8	,,	—	—	34·1*
9	Non-lactating	13·0	3·2	−9·8
10	,,	21·5	15·6	−5·9
11	,,	16·0	9·1	−6·9
12	,,	7·4	0·0	−7·4
13	,,	—	—	−7·3*

* As percentage jugular.

Discussion.

Blood-sugar values for lactating cattle have been recorded by previous workers. The values and methods used are as follows: Hayden and Fish [1923] 46–52 mg. glucose per 100 cc. blood (Benedict); Widmark and Carlens [1925] 60 mg. (Bang and Hagedorn-Jensen); Hayden and Scholl [1928] 52 mg. (Folin-Wu); Little, Keith and Fawns [1928] 75 mg. (Hagedorn-Jensen). Our results would indicate that about 30 % of the material estimated by former workers is to be accounted for by non-sugar reducing substances.

The amounts of non-sugar reducing substances present in samples of blood taken simultaneously from different parts of the animal body do not appear to be equal. Such inequalities, we have found, are of sufficient magnitude to give erroneous estimates of the sugar absorbed by the mammary gland when only total reducing power of the blood is measured. This source of error is avoided when estimates are based on the true sugar content of blood as is done in Table I.

The values we have obtained for true sugar are very variable, ranging from 24 to 60 mg. glucose per 100 cc. blood. This is not surprising when it is remembered that there are four distinct phases in the life cycle of the dairy cow; (1) non-lactating and non-pregnant, (2) non-lactating and pregnant, (3) lactating and non-pregnant and (4) lactating and pregnant. At the present time there are no data available as to the variation of blood-sugar level throughout pregnancy and lactation, but it is hardly to be expected that the value would remain constant during periods which physiologically are so widely different. The variation in blood-sugar level obtained by us may therefore be due to the animals being in different stages of the life cycle, a point which is receiving further attention.

Turning now to differences in the true sugar contents of the bloods taken from different sources in the same animal, we see at once that they are greater than can be accounted for by the modifying influence of salivary secretion and are independent of differences in the amounts of non-sugar reducing substances present. In non-lactating animals the sugar content of the jugular blood is lower than that of the arterial blood, a circumstance which is to be ascribed to the withdrawal from the blood of the sugar which is utilised in the metabolic activities of the tissues of the head and neck. In such animals, however, the sugar content of the mammary venous blood, while usually less than that of the arterial blood, is always greater than that of the jugular blood. It is to be concluded that the sugar required by the inactive mammary gland for oxidative purposes is less than that required by the tissues of the head and neck.

In lactating animals, however, it will be observed that the sugar content of the mammary venous blood is lower than that of the jugular blood and very much lower than that of arterial blood. The question therefore arises as to whether this great lowering of mammary venous blood-sugar in lactating animals represents absorption of glucose by the mammary gland for the pur-

pose of meeting increased energy requirements during secretion, or for the synthesis of lactose. Nothing is known of the energy requirements of the mammary gland, but interesting and relevant facts have been discovered for other secretory organs. Barcroft and Brodie [1905], for example, have shown that the respiratory quotient of the active kidney is unity, representing oxidation of carbohydrate only. Barcroft and Piper [1912] have shown that the oxygen consumption of the resting submaxillary gland of the cat is about 0·02 cc. of oxygen per g. per minute, while in the active state it is as much as 0·084 cc. of oxygen per g. per minute. Anrep and Cannan [1922] report that the resting submaxillary gland of the dog utilises about 2·1 mg. glucose per g. of tissue per hour. In the active state much more sugar is utilised, an additional quantity of about 1·5 mg. glucose being required for each cubic centimetre of saliva secreted.

From these observations there is reason to believe that the energy requirements of the mammary gland will be greater in the active than in the inactive state. Part of the increased sugar absorption must therefore be ascribed to an increased demand for energy-producing substances while secretion is in progress. It is hardly conceivable that this would account for all the sugar absorbed, although this is a point which can only be settled by an investigation of the gaseous metabolism of the mammary gland in a resting and an active state. It seems justifiable therefore to conclude that part of the sugar absorbed by the active mammary gland is utilised in the synthesis of lactose.

It will be observed that the amounts of sugar absorbed by the mammary glands of different animals are widely different. This is important in certain aspects of milk production, since it would clearly be of interest to determine if high sugar absorption could be correlated with high lactose secretion and high milk yield. For this purpose an accurate method of determining the amount of sugar actually absorbed for the purpose of lactose synthesis is desirable. Our results indicate that until knowledge of the energy requirements of the mammary gland is available, comparative blood-sugar analyses alone are not a reliable guide.

Summary.

In non-lactating animals the sugar content of the jugular blood is lower than that of the mammary venous blood, which is itself lower than that of arterial blood.

In lactating animals the sugar content of jugular blood is higher than that of mammary venous blood but lower than that of arterial blood.

The great lowering of mammary venous blood-sugar in lactating animals is to be ascribed partly to the increased energy requirements of the active gland and partly to lactose secretion.

On account of the lack of knowledge of the energy metabolism of the mammary gland comparative blood-sugar analyses alone have been shown to be unreliable guides to lactose secretion.

In conclusion we have to thank Dr N. C. Wright for his unfailing help and criticism throughout this work and Mr L. Jordan, who undertook the blood sampling. One of us (J.H.B.) is indebted to the Trustees of the Carnegie Research Fund for a scholarship during the tenure of which this work was carried out.

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XLIII. THE AMINO-ACIDS OF TISSUES.
VI. DETERMINATION OF THE BASIC AMINO-ACIDS IN SMALL QUANTITIES OF PROTEIN.

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In order to analyse the protein of material which can be obtained only in very small quantities, it became necessary to develop suitable micro-chemical technique. Micro-chemical methods cannot always be a small scale copy of ordinary procedure, as errors in a method which are relatively insignificant when large amounts of material are employed, may be of sufficient magnitude to vitiate micro-chemical work altogether. This is particularly true of the Van Slyke method of estimating the basic amino-acids. The ordinary technique of this method has undergone various minor modifications since its description by Van Slyke [1911], but is recognised as not yet being absolutely perfect. Such errors as have not yet been overcome, or which perhaps are not yet realised, might well become accentuated in a micro-adaptation, so that before it is safe to apply the general outline of this method to a small scale, some of these errors demand careful consideration from the point of view of the general principles of micro-analysis, some of which have been clearly set forth by Pregl [1930].

Narayana and Sreenivasaya [1928] proposed a micro-modification of this method, in which they appear to have recognised that one difficulty would be the estimation of arginine. Plimmer [1916] showed that when arginine is estimated by boiling with caustic alkali, histidine may be decomposed to the extent of some 3 %. Such decomposition, although insignificant when examining most proteins in the usual way would give rise to too great an error for micro-chemical work. The difficulty with the suggestion of Narayana and Sreenivasaya, who use arginase extracts, lies in the fact that these extracts may be very far from pure, and that a number of blanks must be carried out to correct for ammonia liberated by these extracts. Work proceeding in this laboratory makes it clear that complete separation of histidine and arginine is not secured by means of flavianic acid to a sufficient degree of accuracy, when using very small quantities of protein material. We decided, therefore, that a direct determination of histidine would be more dependable. Plimmer and

Phillips [1924] have described a method for the direct determination of histidine by bromination during the ordinary Van Slyke process. They assumed that tryptophan would be destroyed sufficiently to prevent its causing appreciable error by absorbing bromine. Our own results, reported below show that whilst this is the case in the ordinary way, it is not so when small quantities are analysed. We have found that prolonged hydrolysis with acid is required in order that complete destruction of tryptophan may be achieved. When this destruction is incomplete, the diamino-fraction absorbs a greater quantity of bromine than would be required by the amount of histidine present.

The matter of prolonged hydrolysis was of special interest to us in view of the previous observations of one of us [Rosedale, 1929; Rosedale and Morris, 1930] that different tissues required different lengths of time for hydrolysis if concordant duplicates were to be obtained. Our present experiments have led us to the conclusion that bromination of histidine cannot be employed in the micro-procedure. Da Silva [1931] has shown, however, that histidine may be estimated in small quantities of protein by precipitation with mercuric sulphate after alkaline hydrolysis. We have therefore adopted the procedure of hydrolysing separate samples with acid and with alkali and estimating histidine by the latter process. An additional step in routine is usually unwelcome if a greater expenditure of material and time is involved, but we have found that the whole procedure is greatly simplified by the adoption of micro-methods and that it is possible to arrange the work so that no more time is expended than that required for the usual Van Slyke procedure.

Experimental.

Estimation of histidine by bromination. Fifty grams of gelatin were hydrolysed for 40 hours with 25 % HCl. After evaporating to dryness several times *in vacuo*, the hydrolysate was made up to 2 litres. Portions of 100 cc. were used for the Van Slyke determinations which were carried out according to the modification of Plimmer and Rosedale [1925]. The total N of 100 cc. was found to be 0·3416 g. Histidine in the diamino-fraction was also determined by bromination, following exactly the directions of Plimmer and Phillips [1924]. Our blank experiments required 12·2 cc. sodium thiosulphate, of which 1 cc. corresponded to 0·0128 g. of bromine; 10 cc. of the diamino-fraction required 12·0 cc. thiosulphate which shows an absorption of 0·00256 g. bromine and this corresponds to 0·006731 g. of histidine-N. Table I gives the average of 18 experiments.

Table I.

	Arginine-N	Histidine-N	Lysine-N
Ordinary Van Slyke method	0·03453 g.	0·00601 g.	0·2526 g.
By bromination	0·03389 g.	0·006731 g.	0·2518 g.
	Calculated as % of total N.		
Ordinary method	10·1 %	1·7 %	7·3 %
Bromination method ...	9·9 %	1·9 %	7·3 %

There is thus complete agreement between the two methods.

Two and a half grams gelatin + 50 mg. tryptophan were hydrolysed as above. After bromination 11·7 cc. thiosulphate were required, corresponding to an absorption of 0·0064 g. bromine instead of 0·00265 g. which would be expected from the previous experiments on gelatin alone. The extra amount of bromine, while only corresponding to about 1 mg. tryptophan, would cause error if calculated as histidine, as 1 mol. tryptophan absorbs 6 or 7 atoms of bromine and 1 mol. histidine absorbs 2 atoms. [Plimmer and Phillips, 1924; da Silva, 1931.]

Similar experiments were carried out with tryptophan added to gelatin, varying only the time of hydrolysis. It was found necessary to hydrolyse for 160 hours before we could be certain that the tryptophan would cease to exert any influence upon the bromination.

We considered therefore that the behaviour of tryptophan under the conditions of our experiments required further investigation.

Gortner and Holm [1917; Holm and Gortner, 1920] have discussed the formation of humin from tryptophan in the presence of aldehyde or carbohydrate. They divided the humin into three classes, insoluble in acid, soluble in acid, and phosphotungstic humin. They also drew the conclusion from their experiments that the indole portion of the tryptophan is responsible for humin formation, and carried out experiments showing that in the presence of aldehyde, pyrrole may contribute to the formation of humin. While Gortner and Holm do not provide a great deal of information about the phosphotungstic humin, the use of a sintered glass filter for separating the diaminophosphotungstates has brought to our notice the frequency with which an insoluble brown material is left on the filter. As Gortner and Holm point out, some of this material is dissolved by the NaOH during the decomposition of the phosphotungstates. As it seems that the decomposition of tryptophan by reduction may take place in protein hydrolysis, it was essential for us to find out whether such decomposition products as indole and pyrrole could affect the bromination, and thus interfere with the estimation of histidine. 1 % solutions of indole in chloroform and of pyrrole in carbon tetrachloride when brominated according to our usual procedure absorbed 6 atoms of bromine per mol. of indole and pyrrole respectively. The only pyrrolidine compound available was proline and this absorbed no bromine. Since indole and pyrrole may be formed during acid hydrolysis, and since they brominate similarly to tryptophan, it is clear that estimation of histidine in this way would not be possible in a micromethod. Experiments were then undertaken by one of us with the view of determining tryptophan directly, so that its value might be deducted from the histidine. These experiments have already been reported [da Silva, 1931], and although they showed that an accurate estimation of tryptophan could not be assured, a direct estimation of histidine appeared to be possible.

The Van Slyke micro-procedure. As already stated, we determine histidine after hydrolysing with alkali. Acid hydrolysis is carried out upon a separate

sample of material, and from the two sets of data, information which is ordinarily given by Van Slyke's method is secured.

Hydrolysis with alkali. An amount of substance corresponding to 0·3 g. protein is placed in a small Jena glass flask and hydrolysed with 20 % NaOH for 18–20 hours. In accordance with the directions of Folin and Ciocalteu [1927] the alkali is added in the proportion of 4 g. NaOH per g. protein. A few drops of butyl alcohol are added and the boiling is carried out in an air-bath. At the end of the period the condenser is removed and the butyl alcohol boiled off. The solution is then neutralised by adding, drop by drop, a solution of 14 *N* H_2SO_4, and allowing to cool. The hydrolysate is then made approximately normal with respect to H_2SO_4. After filtration, the precipitate is well washed with *N* H_2SO_4. If the washing is sufficiently thorough loss does not occur, and the final volume of the solution and washings amounts to 25–30 cc. After making exactly normal with H_2SO_4, the solution is placed in 15 cc. centrifuge-tubes, about 8 cc. to each tube. To each tube 1 cc. of a 15 % $HgSO_4$ solution is added and after standing for 2–3 hours the tubes are centrifuged and the supernatant liquid is decanted. The precipitate is well washed with a 1·5 % solution of $HgSO_4$, again centrifuged and the supernatant liquid discarded. The precipitate is stirred up with *N* HCl, 10 cc. being added to each tube, and after warming in the water-bath for 5 minutes the mercury precipitate is decomposed with H_2S. The tubes are again centrifuged and the liquid is filtered through a small filter, washing being carried out with 0·1 *N* H_2SO_4. The filtrate and washings are concentrated and made up to 5 cc. in a graduated flask.

Two cc. of this solution are used for the determination of amino-N, using the micro-form of Van Slyke's apparatus, and 1 cc. is used for the determination of total N by Pregl's Kjeldahl method [1930]. In view of the likely presence of some tryptophan in this solution, oxidation must be prolonged, and mercuric sulphate must be added to the oxidising tube.

Histidine is then calculated from the data by the formula

$$\text{Histidine-N} = 3\ (\text{Total N}) - 6\ (\text{Amino-N})$$ [da Silva, 1931].

Hydrolysis with acid. An amount of material equal to the quantity used for the alkaline hydrolysis is hydrolysed with 25 % HCl for 36 hours. After this time the hydrolysate is concentrated 5 or 6 times *in vacuo* in order to remove as much of the HCl as possible.

Determination of the amide-N and humin-N. The acid hydrolysate is made alkaline with the smallest quantity of finely ground CaO and distilled *in vacuo* in a 50 cc. Claisen flask to remove ammonia. In carrying out this procedure we have used a silver condensing-tube as used by Pregl for the micro-Kjeldahl distillation. Instead of using standard alkali for the titration, we have distilled the ammonia into 2 cc. of a saturated solution of boric acid, using bromocresol purple as indicator. The ammonia is then titrated directly with 0·01 *N* HCl. The residue from the distillation is filtered through a 4 cm. paper and well washed with water. The humin-N is determined by estimating the

total N in the precipitate and paper. In carrying out this, and all determinations of total N, the method of Pregl [1930] has been followed exactly, except that we use the boric acid solution instead of standard alkali as stated above. This does away with the necessity of using quartz receivers and boiling off the CO_2 which is necessary in the procedure as described by Pregl, and also does away with the frequent preparation of the standard alkali, a necessity imposed upon us by climate owing to the growth of moulds. In applying Pregl's method to this work it is necessary to take care that the oxidation is sufficiently thorough.

Precipitation with phosphotungstic acid. After many experiments we have found it desirable to add 1 cc. HCl to the filtrate from the humin and to concentrate to a volume just below 10 cc. 1·5 g. nitrogen-free phosphotungstic acid are then dissolved in the minimum of water and added to the solution which is made up exactly to 10 cc. and heated as described in the ordinary procedure in order to dissolve the phosphotungstates so as to obtain a precipitate which is easy to filter. This heating only takes a few minutes, after which the precipitate is allowed to stand for 48 hours.

Treatment of the phosphotungstates. The precipitate is collected on a Jena sintered-glass funnel (G 4 size) of 1 cm. diameter and washed 5 times with 1 cc. 10 % HCl. The filtrate is finally filtered again through a 4 cm. folded paper into a 25 cc. measuring flask and made to volume. The precipitate of the diamino-phosphotungstates upon the filter is dissolved in the smallest possible quantity of 2 *N* NaOH, the filter being washed with water and sucked dry. The solution is then filtered through the same folded paper as used in filtering the mono-amino-portion, into a 10 cc. graduated flask, and made to mark. Estimations of total N and amino-N are made upon both the diamino- and the monoamino-portions. Total N is estimated in 1 cc. portions and amino-N in 2 cc. portions.

From the data secured in this way, knowing the amount of histidine-N from the alkaline hydrolysis, the arginine-N and lysine-N can be calculated.

Determination of cystine. When cystine is present it must be estimated. Plimmer and Lowndes [1927] find that about 40 % of the total cystine is precipitated by phosphotungstic acid. If it is proposed to weigh the sulphur as $BaSO_4$, the phosphotungstic acid must be got rid of. This, together with the fact that we are dealing with solutions of the diamino-acids, and not with solid substances, has caused Pregl's method of sulphur estimation to be rather inconvenient and, in view of the fact that cystine is present in proteins like caseinogen to so small an extent as 0·2 %, renders the method impracticable in such work. We have accordingly attempted estimations of sulphur upon the acid hydrolysate direct, before precipitating with phosphotungstic acid, and have carried out our oxidation by boiling with a mixture of 3 parts HNO_3 and 1 part $HClO_4$ according to a method described by Chatron [1931]. Boiling is carried to dryness in a small flask, and the heating continued for a short time in order to complete the oxidation. After cooling we have dissolved the residue in a few cc. of water and transferred it to a 7 cm. glass dish, precipitated with $BaCl_2$ and proceeded with the technique as described by Pregl.

One cc. of a solution containing 1 mg. cystine per cc. was added to 2 cc. of a gelatin hydrolysate from our first experiments described above. This corresponds to 0·2 % cystine in the gelatin. After carrying out the above procedure we obtained on the first occasion, 1·874 mg. $BaSO_4$, on the second, 1·863 mg. so that our errors are − 1·0 % and − 1·5 %, which would doubtless be increased had we attempted to estimate some 40 % of such a small quantity in a diamino-fraction, after removal of phosphotungstic acid. We have contented ourselves, therefore, with attempting micro-sulphur estimations upon the proteins used in our experiments below, but have failed to secure amounts of $BaSO_4$ that could be weighed upon a micro-balance. We have therefore neglected cystine in these experiments. We consider that it would be important to improve the method for cystine estimation before our general micro-method could be applied to keratins, although we believe that any error involved by its neglect in the estimations of ordinary tissue proteins is insignificant. We hope to return to the study of this point on a future occasion.

Experiments with proteins. Our first experiments using the micro-method were carried out with gelatin and caseinogen as their composition is well known, and could therefore be used as a check upon the method.

Acid hydrolysis: 13·17 g. gelatin were hydrolysed for 36 hours with 25 % HCl. After concentration *in vacuo* several times, the residue was dissolved in water and made up to 100 cc. Total N was 21·0833 mg./cc. The micro-procedure was carried out as already described on 1 cc. portions, and the results are given in Table II.

Alkaline hydrolysis: 135 mg. gelatin were hydrolysed with NaOH and the

Table II. *Percentage of total N.*

			Diamino-fraction					Monoamino-fraction		
	Amide-N	Humin-N	Total N	Amino-N	Arginine-N	Histidine-N	Lysine-N	Total N	Amino-N	Total
Gelatin	1·48	0·16	17·46	11·44	7·41	0·69	9·35	73·29	60·59	92·31
	1·48	0·15	17·68	12·33	6·52	0·69	10·46	74·04	66·07	93·37
	1·50	0·18	22·18	12·33	12·50	0·69	8·97	73·03	63·07	96·89
Mean...	1·48	0·16	19·1	12·03	8·81	0·69	9·59	73·45	63·22	94·19
Caseinogen	11·16	1·16	27·0	18·54	3·45	8·85	10·08	63·24	53·49	102·1
	10·04	1·00	23·1	12·79	5·99	8·85	8·35	63·79	53·44	98·54
Mean...	10·60	1·08	25·0	15·66	4·72	8·85	9·21	63·51	53·45	100·32
Oysters:										
2nd pepsin digest	3·11	11·44	23·36	11·52	10·33	3·79	5·47	57·82	57·80	95·75
	3·12	11·15	23·49	11·62	12·44	3·79	7·24	57·83	57·75	95·68
Mean...	3·11	11·29	23·42	11·57	11·38	3·79	6·35	57·82	57·77	95·71
3rd pepsin digest	5·70	14·49	22·59	11·38	14·01	2·06	4·72	57·32	27·47	100·1
	5·66	14·63	23·64	11·89	13·82	2·06	7·74	57·34	27·68	101·2
Mean...	5·68	14·56	23·61	11·63	13·91	2·06	6·23	57·33	27·57	100·6
Trypsin digest	6·26	3·66	18·50	7·32	6·96	8·0	3·53	71·55	34·18	99·97
	5·79	3·66	25·07	13·37	7·87	8·0	8·58	74·62	64·01	109·14
Mean...	6·02	3·66	21·78	10·34	7·41	8·0	6·05	73·08	49·09	104·05
Torula utilis	5·06	10·79	22·79	7·09	16·36	4·93	1·36	58·99	53·09	97·66
	5·05	10·71	22·79	7·14	16·49	4·93	1·37	58·99	53·13	97·56
Mean...	5·05	10·75	22·79	7·11	16·42	4·93	1·36	58·99	53·11	97·61

procedure described above followed. The results are given for histidine in Table II.

Caseinogen was treated similarly. 16·187 g. caseinogen were used for the acid hydrolysis. After making up to volume the total N was 25·900 mg./cc. The results of these experiments have been collected in Table II.

For the alkaline hydrolysis, 171 mg. were used. Total N of the histidine portion = 2·703 mg. Amino-N = 0·9478 mg. From the formula [da Silva, 1931] histidine-N = 2·423 mg., consequently the amount of histidine-N calculated for the acid hydrolysis is 2·293 mg.

It will be seen from Table II that the same degree of concordance between duplicates as is usually obtained with Van Slyke's method may be secured with our procedure.

We have used this method for a further examination of oysters. Rosedale and Morris [1930] recorded data for oysters, their data being obtained from the filtrate of a peptic digest. It was of interest to see how complete the digestion had been, and also to note the difference, if any, between the types of amino-acids that took longer to be released by pepsin. Rosedale and Morris had found in the first peptic digest a large amount of histidine. We therefore submitted the residue from their peptic digest to two further digestions with pepsin, each lasting 17 days, filtering off the solid material and using the filtrate for our experiments. The residue was finally digested with trypsin. These three digests have been submitted to our micro-procedure and our results are given in Table II. As Rosedale and Morris had already made one peptic digest with this material, we have called our first digest "pepsin 2."

Opportunity has been taken of examining the protein of *Torula utilis*. The organism was grown upon the following medium which is free from organic nitrogen.

Glucose	50·0 g.	$MgSO_4, 7H_2O$	0·50 g.
$NaNO_3$	2·0 g.	$FeSO_4, 7H_2O$	0·01 g.
KH_2PO_4	1·0 g.	Agar-agar	20–30 g.
KCl	0·5 g.	Water	1000 cc.

After incubation the cells were washed with saline, then water, and after centrifuging were dried over $CaCl_2$. An analysis gave the following results: nitrogen 7·05 %, ash 1·09 %, fat 0·01 %, moisture 80·03 %. The dried material was extracted with 1 % acetic acid and digested with pepsin in the usual way. The filtrate from the peptic digest was used for our experiments. Quantities of 300 mg. of the dried material were weighed out and digested for each experiment. The micro-procedure as detailed above was carried out and the results are given in Table II.

SUMMARY.

1. A micro-method for the estimation of the basic amino-acids is described.

2. Data secured by its use upon gelatin, caseinogen, oysters and *Torula utilis* are presented.

3. Some further evidence of the probable interference of tryptophan with the Van Slyke method has been brought forward.

We desire to express our thanks to Prof. H. Raistrick for giving us the culture of *Torula utilis* and for advice upon the method of culturing it. Prof. W. A. Young has kindly undertaken the culturing for us. Grateful acknowledgement is also made to the Colonial Development Fund for a grant which has defrayed in part the expenses of this investigation.

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XLIV. THE INTERPRETATION OF THE COLOUR MATCH IN THE ANTIMONY TRICHLORIDE TEST FOR VITAMIN A.

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In a recent paper, Coward *et al.* [1931] compared the vitamin A potencies of 11 cod-liver oils and 2 concentrates, as biologically determined, with (amongst other things) the blue value of the oil determined directly and indirectly *via* the unsaponifiable matter. It was found that the blue value determined *via* the unsaponifiable matter gave better agreement with the biological determination of vitamin A than did the blue value determined directly.

It is generally observed that for oils of medicinal grade, the blue value determined *via* the unsaponifiable matter is higher than the blue value determined directly, while for very potent fish-liver oils the blue values determined directly and indirectly are in approximate agreement.

In the present paper, evidence is presented that the discrepancy between the direct and indirect determinations of the blue values of oils of medicinal grade is to be accounted for by the method of interpretation of the colour match.

The blue colours given by cod-liver oil and concentrates in the antimony trichloride reaction vary quite widely in quality. For a cod-liver oil of medicinal grade, a typical Lovibond colour match is

5·5 blue + 0·5 yellow,

while for a preparation of unsaponifiable material a typical match is

5·5 blue + 2·5 yellow − 0·8 neutral.

Exceptionally potent cod-liver oils and other liver oils give blue colours similar in quality to that given by unsaponifiable material, but various samples can be found to give colours intermediate in quality between the two examples given, and certain oils (usually old or oxidised samples) give reddish colours, such as

5·5 blue + 0·2 yellow + 1·0 red.

According to the usual interpretation the three colour matches given above represent equal amounts of chromogen, since only the value of the blue component is considered.

It seems reasonable to suppose that the preparations containing the highest concentration of chromogen give the colours nearest to what would be

given by the uncontaminated chromogen itself. That is, the colour given by a concentrate or a very potent oil is mainly due to the chromogen which it is required to estimate, and the colours given by oils of medicinal grade are different in quality because of some interfering factor or factors. The difference between the three colour matches given above is essentially a difference of redness, and it can be shown that the colour given by medicinal cod-liver oil can be reproduced by the addition of a red glass to the colour given by a concentrate. According to Heilbron, Gillam and Morton [1931] the cod-liver oil environment causes a displacement of the absorption bands of the chromogen itself towards the blue end of the spectrum. Thus the colour given by cod-liver oil is more reddish than that given by a concentrate.

Experiments with copper sulphate solution.

To investigate the effect of varying colour quality on the blue component in the colour match, some experiments have been made with a copper sulphate solution observed through Lovibond red glasses of various values. The actual colour matches (in each case the average of the readings made by two or three observers) are given in Table I.

Table I.

Copper sulphate solution covered by a red glass of value	Colour match				Blue *minus* yellow
	Blue	Yellow	Red	Neutral	
6·0	3·83	0·23	2·13	—	3·60
5·0	3·85	0·25	1·1	—	3·60
4·0	4·06	0·36	0·53	—	3·70
3·0	4·2	0·5	0·1	—	3·7
2·0	4·5	0·9	—	—	3·6
1·0	5·45	1·75	—	−0·55	3·70
0·0	6·9	3·33	—	−1·13	3·57

These colour matches cover a range wider than that usually found in cod-liver oils and concentrates, but matches such as those given by the copper sulphate solution covered by Lovibond red glasses of 3·0, 2·0 and 1·0 are frequently encountered. It is observed that the value of the blue component increases throughout, and very rapidly in the last three matches.

This variation of the blue value with the redness of the combination of copper sulphate solution and red glasses is not surprising, for, since the neutral glass consists of red, yellow and blue glasses of equal value bound together, the effect of the progressive removal of red on the first colour match of Table I can be calculated (on the assumption that the glasses are additive) as follows.

Table II.

$$(3{\cdot}83 \text{ blue} + 0{\cdot}23 \text{ yellow} + 2{\cdot}13 \text{ red}) - 1 \text{ red} = 3{\cdot}83b + 0{\cdot}23y + 1{\cdot}13r.$$
$$\text{,,} \quad \text{,,} \quad \text{,,} \qquad - 2 \text{ red} = 3{\cdot}83b + 0{\cdot}23y + 0{\cdot}13r.$$
$$\text{,,} \quad \text{,,} \quad \text{,,} \qquad - 3 \text{ red} = 4{\cdot}7b + 1{\cdot}1y - 0{\cdot}87 \text{ neut.}$$
$$\text{,,} \quad \text{,,} \quad \text{,,} \qquad - 4 \text{ red} = 5{\cdot}7b + 2{\cdot}1y - 1{\cdot}87 \text{ neut.}$$
$$\text{,,} \quad \text{,,} \quad \text{,,} \qquad - 5 \text{ red} = 6{\cdot}7b + 3{\cdot}1y - 2{\cdot}87 \text{ neut.}$$
$$\text{,,} \quad \text{,,} \quad \text{,,} \qquad - 6 \text{ red} = 7{\cdot}7b + 4{\cdot}1y - 3{\cdot}87 \text{ neut.}$$

When there is no red glass in the colour match, the effect of removing red is to add blue + yellow − neutral in equal proportions. By comparing Table II with Table I it is seen that the value of the blue component would be expected to increase even more rapidly than is actually found.

From Table I it is evident that the value of the blue component could not be taken as an index of the amount of copper present in a solution whose colour quality varied in the same way as the blue solutions given by cod-liver oils and concentrates vary. The amount of copper in the solution used in the above matches remained constant, but the value of the blue component did not, and as shown in Table II, it could not be expected to. The only simple expression derived from the colour match which could be expected to remain constant is the value "blue *minus* yellow," and reference to Table I shows that this is found to remain practically constant independent of the redness of the combination of copper sulphate solution and red glass. The highest value found was 3·7 and the lowest 3·57, a difference well within the limits of error in carrying out the matches.

"*Blue* minus *yellow*" *values of cod-liver oils and concentrates.*

From the above considerations it would be expected that the value "blue *minus* yellow" would furnish a better measure of the chromogen of cod-liver oil than the blue value alone.

An experiment was made in which ergosterol, which gives a red colour with antimony trichloride solution, was added to a vitamin A concentrate. The match of a 0·25 % solution of concentrate was

7·5 blue + 3·0 yellow − 0·8 neutral.

A 0·1 % solution of ergosterol gave with the antimony trichloride reagent a red colour increasing in intensity. The match at about 20 seconds was

2·0 red.

The match of the two solutions in superimposed cells, or in a solution containing together the same amounts of the concentrate and ergosterol as were used in the separate matches, was

5·0 blue + 0·7 yellow.

The addition of the red chromogen lowered the blue value of the concentrate in the ratio 7·5/5, but the value for "blue *minus* yellow" remained practically unchanged.

Just as the blue value of a concentrate is lowered by the addition of a red chromogen, so the blue value of a cod-liver oil is increased by the removal of redness on rejection of the saponifiable material. The blue value of an oil was determined directly by the method recommended by the General Medical

Council [1931] and indirectly *via* the unsaponifiable matter. The results expressed in full were:

Colour match of original oil calculated to 20 % wt./vol. solution:	Colour match on unsaponifiable matter calculated to 20 % wt./vol. solution of original oil:
11·8 blue + 1·25 yellow.	14·7 blue + 4·25 yellow − 1·18 neutral.
Blue value = 11·8.	Blue value = 14·7.
"Blue *minus* yellow" value = 10·55.	"Blue *minus* yellow" value = 10·45.

The decrease of redness on saponification and extraction causes a rise in the blue value in the same way as the removal of red from the combination of copper sulphate and red glasses causes a rise in the blue component of the colour match, and in both cases the value for "blue *minus* yellow" remains practically unchanged.

I have been able to examine oil A and oil L in the series assayed by Coward *et al.* [1931]. The colour matches determined were:

Oil A. 0·2 cc. of a 10 % solution:	Oil L. 0·2 cc. of a 0·35 % solution:
5·5 blue + 0·4 yellow.	5·5 blue + 2·5 yellow − 0·8 neutral.
Blue value = 11·0.	Blue value = 314.
"Blue *minus* yellow" value = 10·2.	"Blue *minus* yellow" value = 171.

The ratio of the blue values of these oils is oil L/oil A = 28·5/1, and the ratio of the "blue *minus* yellow" values is 16·8/1. The ratio of their biological values is given in Coward's paper as 15·6/1.

Summary.

It is known that the vitamin A potencies of very potent oils and concentrates compared with a medicinal oil as standard are lower than would be expected from their blue values. It is also known that the blue value of a medicinal grade oil determined *via* the unsaponifiable fraction is higher than the blue value determined directly. These discrepancies are attributed to the influence of varying colour quality on the blue component of the colour match. It is shown that the blue reading in the Lovibond match of a copper sulphate solution varies widely as the quality of the light from the solution is varied by the interposition of red glasses. The variation obtained is similar to that given in the colour matches on a wide range of oils and concentrates.

It is also shown that the value for "blue *minus* yellow" can be expected to remain constant however the redness of the solution varies, and it is considered that the value "blue *minus* yellow" would be a better criterion of vitamin A than the blue value as usually read. Biological tests and the fact that the "blue *minus* yellow" value of an oil determined *via* the unsaponifiable fraction agrees with the value determined directly support this conclusion.

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XLV. THE RELATIONSHIP BETWEEN SUGAR IN BLOOD AND LACTIC ACID IN BRAIN.

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AND
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(*Received February 24th, 1932.*)

In a series of earlier communications [1925, 1, 2; 1927] Holmes and Holmes developed the hypothesis that the brain tissue of the living animal was dependent for the supply of carbohydrate from which it formed lactic acid on glucose supplied to it by the blood stream. Since that time, several workers [Kinnersley and Peters, 1929, 1930; Jungmann and Kimmelstiel, 1929] have, on the basis of their own experiments, questioned the validity of this view. If the hypothesis originally advanced by Holmes and Holmes is the correct one it emphasises the dependence of the brain upon the circulation, not only for oxygen, but also for carbohydrate, and it renders it possible that the train of symptoms shown by the hypoglycaemic animal may be referred directly to deficiency of carbohydrate supply to the cerebral tissue, just as syncope is the result of anoxaemia of those same cells.

It was in the hope of reaching a definite conclusion that the experiments here reported were undertaken: they constitute a re-investigation of the original hypothesis, and we believe that they succeed in demonstrating its validity.

In the original experiments, Holmes and Holmes employed rabbits and cats. The animals were killed, the brains removed, cooled as quickly as possible with ice and salt freezing mixture, and their lactic acid contents compared with the level of the blood-sugar at the time of killing. Since only small changes in lactic acid or carbohydrate content could be observed when the tissue was incubated under anaerobic conditions, it was concluded that the brain contained little or no lactic acid precursor. It was however recognised that the so-called resting values might in reality be "final" values, since 3–4 minutes necessarily elapsed after killing before the brain could be cooled, and a considerable amount of lactic acid formation might take place in that time. Since the original experiments have been performed, the work of McGinty and Gesell [1925], Kinnersley and Peters [1929] and Jungmann and Kimmelstiel [1929] has shown that this was, in fact, the case. These authors, by cooling the tissue in liquid air, obtained "resting" values lower than those of Holmes and Holmes. These low initial values however increased on incubation to values of the same

order as those observed by the last-named workers, all of whose figures represented, evidently, "final" or nearly "final" values. On these grounds, both Kinnersley and Peters and Jungmann and Kimmelstiel have concluded that the brain contains lactic acid precursor, and therefore are unwilling to admit its dependence on the blood-sugar in this respect. They do not appear however to have attempted to investigate the actual relationship between blood-sugar and brain-lactic acid, that is to repeat the experiments upon which the original hypothesis was founded.

In our re-investigation of the matter, we employed mice. Blood-sugar was lowered when required by insulin and raised by intraperitoneal injections of glucose. Liquid air was used to fix the tissue for the purpose of obtaining "initial" values.

Method.

Mice were used, in groups of six or eight, for each experiment. Low blood-sugar values were secured by injecting insulin after a preliminary period of starvation. For normal values the animals were taken directly from stock. For high values the animals were given an intraperitoneal injection of glucose solution of appropriate strength 15 minutes before being killed. The mice were decapitated with a single stroke of a pair of strong, sharp scissors. Four (or if six animals only were used, three) of the heads dropped directly into a flask of liquid air. They were frozen within 2–3 seconds of decapitation, and the brains were subsequently removed, and worked up for "initial" values. The remaining heads were placed in an incubator at 37° for periods of 1 hour, or sometimes of 30 minutes. Blood was collected from each severed trunk in turn in a small dish containing oxalate: a "pooled" sample from the whole group of animals was thus obtained, and its sugar content determined by the method of Hagedorn and Jensen [1923].

The tissue was worked up in the same way for both sets of values, the method differing somewhat according to the substance or substances to be estimated.

Lactic acid only. The brains were removed from the skulls and weighed. They were then ground with sand in a mortar with 5 cc. of 20 % trichloroacetic acid, and the "brei" poured into a 25 cc. volumetric flask. The mortar was washed out, and the washings, *plus* 10 cc. more of the 20 % trichloroacetic acid, were added to the flask. The contents were made up to volume, and filtered. An aliquot part of the filtrate was treated with 10 % copper sulphate and a 10 % lime suspension in the usual way and filtered, and a suitable volume of filtrate taken for lactic acid estimation by the method of Friedemann, Cotonio and Shaffer [1927].

Lactic acid and total carbohydrate. The method used was based on that of Carruthers [1931]. After weighing, the tissue was ground with 4 cc. of boiling water, and washed into a 15 cc. volumetric flask with two more 3 cc. portions of boiling water. 3·5 cc. of 40 % trichloroacetic acid were added, and the con-

tents of the flask made up to the mark with hot water. After standing for 30 minutes, the material was filtered. 10 cc. of filtrate were pipetted into 25 cc. centrifuge-tubes; 0·66 cc. of conc. HCl was added, and the tubes were heated (with air condensers) in a boiling water-bath for 3 hours. After hydrolysis the fluid was transferred to a 15 cc. flask; 1·0 cc. of 10 % copper sulphate was added, and the fluid neutralised with concentrated sodium hydroxide. After the addition of 2·0 cc. 10 % suspension of lime, the contents of the flasks were made up to volume, transferred to centrifuge-tubes, cooled for 30 minutes in ice and centrifuged. 10 cc. of the supernatant fluid were taken for lactic acid estimations: the precipitate was used for estimation of carbohydrate, by the technique previously described [Holmes and Ashford, 1930]. This method, as is claimed by Carruthers, permits the estimation of the total carbohydrate of the tissue. The hot water extracts "soluble carbohydrate" and glycogen; the trichloroacetic acid precipitates proteins and lipoids. The glycogen having been hydrolysed during the period on the water-bath, the sugar arising from it, together with other carbohydrate, is separated from other substances by the "copper-lime" procedure.

Glycogen. This substance was estimated by the method described by Holmes and Ashford [1930]. The tissue was placed at once in absolute alcohol. Simultaneous estimations of lactic acid were not carried out.

The results of these experiments are presented in Tables I and II, and in Figs. 1 and 2. While it is desired to give the figures actually obtained, the general results can be most conveniently appreciated by an inspection of the curves. They can be summarised quite briefly, as follows. For all values of blood-sugar, the initial value for brain-lactic acid is low, and very fairly constant; except for the lowest, *i.e.* those obtained in severe hypoglycaemia, the average figure is 67 mg. per 100 g. of tissue. This confirms the findings of McGinty and Gesell, Jungmann and Kimmelstiel, and Kinnersley and Peters, and makes it clear that the initial values previously obtained by Holmes and Holmes were too high. The final values on the other hand show an almost linear relationship to the blood-sugar until the latter reaches a value of about 200 mg. per 100 cc.; as long, that is, as it is within what may fairly be looked upon as a normal range of values. When the blood-sugar level exceeds 200 mg. per 100 cc. the curve falls off rather sharply, and the slope becomes much more gradual. An almost exactly similar relationship is to be seen in the figures published for rabbit and cat brain [Holmes and Holmes, 1927].

The explanation of the shape of the curve for "final" values shown in Fig. 1 is afforded by the curve for "initial" total carbohydrate values in Fig. 2. Curve *A*, Fig. 1, falls away after the value of 200 mg. of sugar per 100 cc. of blood has been reached because at higher levels of blood-sugar the total carbohydrate in the brain no longer follows the blood-sugar value, but itself tends to fall away (Fig. 2 *a*). The formation of lactic acid evidently is limited by the lack of precursor, the amount of which depends (1) on the blood-sugar level and (2) on the ability of the brain cells to pick up carbohydrate

Table I.

Blood-sugar mg./100 cc.	Lactic acid mg./100 g.			Total carbohydrate mg./100 g.			Procedure
	Initial	Final	Rise	Initial	Final	Fall	
34	24	30	6	64	33	31	Starved 15 hrs. 4 units insulin subcutaneously
35	49	43	6	—	—	—	,,
43	—	48	—	—	—	—	,,
48	30·5	47·4	17	—	—	—	,,
121	55	114	59	—	—	—	No injection
137	83	108	25	121	33	88	,,
139	93	121	28	102	56	46	,,
140	59	116	57	—	—	—	,,
197	—	—	—	152·2	43·8	108·4	0·5 cc. 10 % glucose killed after 15 mins.
198	54	153	99	—	—	—	,,
202	79	128	49	—	—	—	,,
263	79	148	69	153	65	88	0·5 cc. 20 % glucose killed after 15 mins.
291	—	—	—	159	62·2	96·8	,,
284	41	168	127	—	—	—	,,
296	62	173	111	221	62	159	0·5 cc. 30 % glucose killed after 15 mins.
297	63	173	110	—	—	—	0·5 cc. 20 % glucose killed after 30 mins.
487	—	—	—	262	50·4	211·6	0·5 cc. 30 % glucose killed after 15 mins.
543	53	185	132	—	—	—	,,
593	67	224	157	210	54	156	0·5 cc. 40 % glucose killed after 15 mins.
618	67	186	119	—	—	—	0·5 cc. 30 % glucose killed after 15 mins.
637	75	193	118	195	78	117	0·5 cc. 40 % glucose killed after 15 mins.
670	—	—	—	225	43	182	,,
844	63	210	147	—	—	—	,,
946	54	189	135	—	—	—	,,

Table II.

Date	Blood-sugar mg./100 cc.	Glycogen mg./100 g. fresh tissue			Injection
		Initial	Final	Fall	
15. i. 32	145	60	40	20	None
26. i. 32	644	33	22	11	None
19. i. 32	653	74	35	39	0·5 cc. 40 % glucose
21. i. 32	880	57	51	6	0·5 cc. 40 % glucose

from the circulating fluid. Curves 1 (*a*) and 2 (*a*) are not superposable, it is true. But there is a considerable difference between the relative simplicity and accuracy of the methods for estimating lactic acid and total carbohydrate respectively. The figures for lactic acid are on the whole surprisingly steady; those for total carbohydrate show a very considerable scatter, and the method which has to be employed is neither short nor simple. Moreover, as has been

pointed out elsewhere, even with the copper-lime technique it is sure that all the material estimated is not glucose.

While these experiments leave little room for doubt that the brain does depend, in a very direct sense, upon the blood-sugar for its lactic acid precursor, they throw no light on the possible steps by which the glucose of the blood is transformed to lactic acid. Ashford and Holmes [1929] have found reason to believe that hexosephosphate formation is not a necessary step in

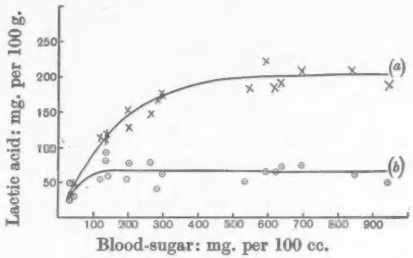

Fig. 1.

⊙—⊙ Initial values ×—× Final values

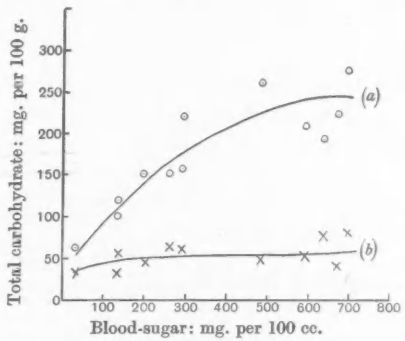

Fig. 2.

⊙—⊙ Initial values ×—× Final values

the production of lactic acid by brain *in vitro*, though it does intervene in the conversion of glycogen into lactic acid. But it is still possible that sugar might be taken from the blood and stored in the living brain as glycogen, to break down rapidly after death.

To test this, glycogen was estimated both in the initial and in the final samples. The results given in Table II, show that

(1) the initial glycogen values are independent of the blood-sugar;
(2) the fall in glycogen on incubation is small compared with the total rise in lactic acid;
(3) the total amount of glycogen in the brain is relatively small.

We are satisfied therefore that glycogen is not an intermediary in the conversion of glucose into lactic acid by the brain.

The "resting" values that we find for lactic acid seem to be independent of the blood-sugar, unless the latter is very low, when they tend to decrease. In such cases, they approximate closely to the final values: there is however always some rise on incubation, which we attribute chiefly to a conversion of some glycogen into lactic acid. It will be noticed that curve *A*, Fig. 1, would cut the ordinate at zero blood-sugar. This is somewhat unexpected, since the method of Hagedorn and Jensen probably gives values appreciably higher than the true glucose content of the blood, and one would expect to find no brain-lactic acid when there was still apparently some sugar in the blood. If, however, as we believe, there is always some glycogen in the brain, no matter how low the blood-sugar may be, this state of affairs may be explained. Curve *A*, of Fig. 2, will in fact cut the ordinates at a level indicating that, at zero blood-sugar, the brain will contain some 35 mg. of total carbohydrate per 100 g.

The "initial" lactic acid value must be the resultant of at least three processes; lactic acid formation, lactic acid removal by diffusion into the blood stream, and lactic acid removal by oxidation *in situ*. It is remarkable that these should balance to give such steady values as they do. It is even more remarkable that such a readily diffusible substance as glucose should apparently fail to be taken up by the brain cells beyond a certain limit of its concentration in the blood. The failure to find an increase in total carbohydrate of the brain cannot be referred to an increased rate of removal of lactic acid (and therefore of sugar) by oxidation, for the oxygen uptake of the brain, even *in vitro*, is for the early period of any experiment almost independent of the lactate concentration [Ashford and Holmes, 1931], showing that the lactic-oxidising enzyme systems immediately after death (and therefore presumably during life) are fully saturated with substrate.

It is evident, both from the figures and the curves, that the fall in carbohydrate is in general greater than the observed rise in lactic acid. In the first few experiments, the reverse appeared to be the case, and a statement to that effect appeared in a previous publication [Ashford and Holmes, 1931]. Little is so far known about the steps whereby glucose is converted into lactic acid by the brain, except that the process does not appear to require the intervention of phosphate, but the discrepancy between these curves suggests that it is probably a complex affair.

While we believe that these results show clearly that the amount of lactic acid precursor in the brain depends upon the blood-sugar, and while they fit well with the lowered O_2 uptake observed for excised brains of hypoglycaemic animals [Holmes, 1930], they do not help us to understand the actual mechanism of hypoglycaemic symptoms, save in so far that it appears from curve 1 (*b*) that at blood-sugar levels at which these symptoms occur there is a definite drop in the true "resting" lactic acid level, *i.e.* in the concentration of lactic acid in the living brain cells. It is conceivable that the function of the

lactic acid is to maintain a stable condition at some intracellular surface, and that its removal results in a profound disturbance of function. This disturbance, of course, does not necessarily show itself in the occurrence of convulsions which, though usual in the hypoglycaemic rabbit, are usually, though not always, replaced by muscular flaccidity and collapse in the mouse, and occur but rarely in the human subject.

SUMMARY.

The relationship between brain-lactic acid and blood-sugar content has been re-examined with the following results.

1. The lactic acid content of mouse brains fixed rapidly in liquid air is invariably low. (This confirms the results of other workers.)

2. The amount of lactic acid formed on anaerobic incubation depends on the blood-sugar level at death, up to blood-sugar values of some 200 mg. per 100 cc.

3. At higher levels there is a smaller increase in lactic acid in proportion to the increase in blood-sugar.

4. This increase is accounted for by a corresponding failure of the brain tissue to take up carbohydrate.

5. At blood levels at which hypoglycaemic symptoms occur, the "resting" brain lactic acid level is decreased.

We would thank Mr T. C. Fletcher for his careful and skilful technical assistance.

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Note added April 4th, 1932. Since this paper went to press, a communication by Haldi [(1932) *Amer. J. Physiol.* **99**, 702] has been published, in which appear figures for the time relationships of lactic acid production by (dog) brain tissue. The initial values found are in substantial agreement with those given here, and the maximum lactic acid production is shown to occur in 15–20 minutes at 38°. The final values reached would correspond, in the mouse, to a blood-sugar level of about 200 mg. per 100 cc.

XLVI. NOTE ON THE SUPPOSED PRODUCTION OF MACLURIN FROM ACACATECHIN.

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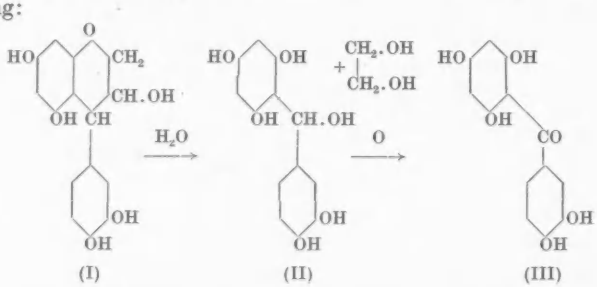
Hazelton and Nierenstein [1924] state that Nierenstein has established the constitution (I) for acacatechin both analytically and synthetically. On the other hand Freudenberg has advanced evidence tending to show that the substance is a mixture of stereoisomerides, namely, *l*-epicatechin, *l*-catechin and *r*-catechin (all corresponding to (IV)). Hazelton and Nierenstein do not accept the findings of Freudenberg, because they observed that maclurin (III) was formed in 86 % yield when *Penicillium solitum* was cultured in a solution of acacatechin containing 5 % sucrose. The production of maclurin from acacatechin is in agreement with the constitution ascribed to acacatechin by Nierenstein and the suggested mechanism of the decomposition was the following:

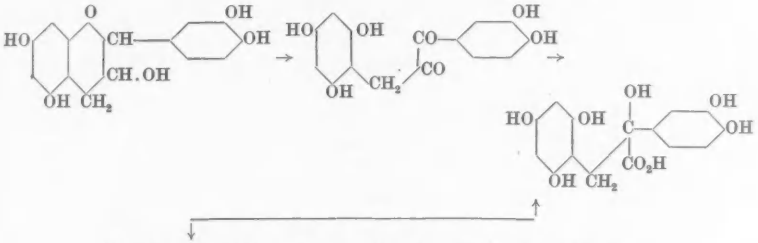


(I) (II) (III)

The leuco-maclurin (II) and glycol were not isolated. It should be noted that the formation of maclurin from acacatechin does not necessarily exclude the Freudenberg formulation (IV) for the latter substance, and Robinson [1928] has suggested that the course of the degradation might involve a double benzil-benzilic acid transformation of the type supposed by some to occur in the formation of citric acid from glucose, as shown on p. 389.

Hazelton and Nierenstein had noticed that a specific mould developed in dilute solutions of catechin and that a coloured precipitate was produced. The mould was identified as *Penicillium solitum* and the precipitate as maclurin. They therefore made a 0·72 % solution of acacatechin in water containing 5 % sucrose, inoculated it with *Penicillium solitum* and kept it in an

incubator at 22°. The first sign of growth became visible after 7 days and after several weeks the solid was collected and afforded pure maclurin after extraction with boiling water and purification through the lead salt.



$C_6H_2(OH)_3CH_2.CO.C_6H_3(OH)_2 \rightarrow C_6H_2(OH)_3.CO.CO.C_6H_2(OH)_2 \rightarrow$
$C_6H_2(OH)_3C(OH)(CO_2H)C_6H_3(OH)_2 \rightarrow C_6H_2(OH)_3CO.C_6H_3(OH)_2$

(IV)

An attempt to repeat these experiments has now been made, but with negative results. Controls using a dilute solution of glucose in pure water containing small amount of inorganic salts were instituted. Although it must be assumed that the normal technique was adopted, Hazelton and Nierenstein do not specifically state that they sterilised their solutions; therefore experiments with both sterilised and unsterilised solutions were made. Different flasks were kept at 22° and 28° in order to determine how the growth of the mould was influenced by the temperature. These variations of the original experiments were carried out with solutions of Gambier catechin rather than with the less accessible acacatechin, and from them favourable conditions for growth were ascertained. A solution of acacatechin was then made and kept under those conditions, and another solution was made and kept under the conditions described in Hazelton and Nierenstein's paper, *i.e.* in 5 % aqueous sucrose solution at 22°.

A yellowish brown solid was obtained from the acacatechin solutions, but it could not be recrystallised from hot water, or dilute acetic acid, or purified through the lead salt, nor did it give a precipitate with bromine in aqueous solution, as did an authentic specimen of maclurin (Schuchardt). It was concluded therefore that the solid was not maclurin.

Experimental.

The catechin employed in most experiments was Gambier catechin (Merck). The *Penicillium solitum* was kindly supplied by Dr Thom of the Laboratory of the Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, to whom the author is greatly indebted. In view of the sequel it ought to be mentioned that strains of *Penicillium* cannot always

be characterised with great accuracy and Dr Thom stated that the specimen was very close to, and probably identical with, *Penicillium solitum*.

The following solutions were made up to 500 cc. and were kept at the temperatures indicated:

					Temp. of incubator
(*A*)	Reeder's solution*	0·72 % of catechin	Inoculated	Sterilised	22°
(*B*)	,,	,,	,,	,,	28°
(*C*)	,,	,,	Not inoculated	,,	22°
(*D*)	,,	,,	,,	,,	28°
(*E*)	,,	No catechin	Inoculated	Unsterilised	22°
(*F*)	,,	,,	,,	Sterilised	28°
(*G*)	,,	,,	Not inoculated	,,	22°
(*H*)	,,	,,	,,	Unsterilised	28°
(*I*)	5 % aqueous sucrose	0·72 % of catechin	Inoculated	Sterilised	22°
(*J*)	,,	,,	,,	Unsterilised	28°
(*K*)	,,	,,	Not inoculated	,,	22°
(*L*)	,,	,,	,,	Sterilised	28°
(*M*)	,,	No catechin	Inoculated	Unsterilised	22°
(*N*)	,,	,,	,,	Sterilised	28°
(*O*)	,,	,,	Not inoculated	Unsterilised	22°
(*P*)	,,	,,	,,	Sterilised	28°

* Reeder's solution contains: ammonium sulphate, 0·3 %; magnesium sulphate, 0·07 %; potassium dihydrogen phosphate, 0·10 %; dipotassium hydrogen phosphate, 0·016 %; sodium chloride, 0·05 %; calcium nitrate, 0·04 %; glucose, 5 %.

The solutions were placed in Pasteur flasks, which were plugged with cotton-wool. To ensure sterilisation these flasks were heated at 100° for 30 minutes on 3 successive days; they were then placed in incubators. In all flasks which had been inoculated with *Penicillium solitum* growth appeared after 8 days, and a yellowish brown precipitate was produced; this did not increase after 8 weeks (found in material dried at 120°: C, 47·5; H, 6·3 %). No growth appeared in the flasks which had been sterilised and not inoculated (*C*, *D*, *G*, *L*, *P*), and a very small growth in the flasks which were not inoculated and unsterilised (*H*, *K*, *O*). No coloured precipitate was formed in any flask which did not contain catechin. On the whole growth was more abundant in the Reeder's solution than in the sucrose, and was favoured by the higher temperature—thus flask (*B*) contained the most solid. After 8 weeks the solid was filtered off from all the solutions, and, as some of the solid adhered to the mould, this was extracted with boiling water.

A brown solution was obtained, but no solid separated on cooling; after concentration to a small bulk a colloidal solution was obtained from which no solid could be salted out. No success attended the attempts to purify this material; maclurin which crystallises from hot water with facility could not be isolated, nor was its presence indicated by the general reactions of the material.

A further set of experiments was carried out with acacatechin. A solution (500 cc.) containing 0·72 % of acacatechin and 5 % of sucrose in water was inoculated with the mould and kept at 22°. Reeder's solution (500 cc.) containing 0·72 % of acacatechin was sterilised, inoculated and then kept at 28°. The precipitate obtained was similar to that produced from Gambier catechin but had a purer yellow colour.

After extraction with boiling water, nothing separated on cooling, and the solution was concentrated to obtain the amorphous product. According to Hazelton and Nierenstein the material may be purified through the lead salt [Hoesch and von Zarzecki, 1917] and accordingly this method was tried without success. A specimen of maclurin supplied by the firm of Schuchardt could be recrystallised from hot water and dilute acetic acid, and had the properties attributed to the substance in the literature.

Summary.

Under the conditions above, and which differ in no obvious respect from those described by Hazelton and Nierenstein, no evidence of the transformation of Gambier catechin or acacatechin into maclurin could be obtained.

The author wishes to thank Dr Thom for supplying the *Penicillium solitum* and Prof. Drummond for his kind interest in the investigation.

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XLVII. THE PROTEINS OF GRASSES.
I. PRELIMINARY COMMUNICATION.

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(*Received February 26th, 1932.*)

Introduction.

A method for the preparation of leaf-proteins was described by one of us some years ago [Chibnall, 1923]. It has since been applied to the leaves of many different types of plants, but not always with success, for proteins in a state of purity were obtained in good yield only from certain herbaceous plants having a leaf cell sap which was alkaline with respect to the isoelectric point of the protein. From other leaves especially of ligneous plants and even of herbaceous plants in which the conditions, such as the p_H of the leaf sap *etc.*, were favourable, the yields of protein were disappointingly small, and the products were impure. At that time no explanation of this difference in behaviour could be put forward [Chibnall and Grover, 1926].

During the past two years we have attempted to use this method to prepare proteins from various grasses. To obtain any appreciable yield of protein it has been necessary to modify it in certain essential details, but even so it appears that the difficulties inherent in preparations of this type have not yet been completely overcome, because we find that a procedure which will give a good yield of protein from a sample of cocksfoot cut in spring will give no protein at all from a similar sample cut from the same plot two or three months later. We feel however that a definite advance in the technique of leaf-protein preparation has already been achieved, and the present paper describes in some detail the modifications of the original method which we now recommend and gives also a preliminary account of our analyses of the proteins of cocksfoot which have so far been prepared.

Experimental.

The preparation of proteins from cocksfoot (Dactylis glomerata).

It will be convenient to describe briefly the original ether method of Chibnall [1923], as experience in its application has enabled us to obtain a clearer conception of the nature of the separation which is obtained between

the protein and the simpler water-soluble nitrogenous products present in the leaf cells.

The leaves are first cytolysed with ether. On cytolysis the cell protoplasts shrink and become freely permeable to the aqueous contents of the vacuoles. The cellulose cell wall is permeable to water, consequently the cells lose their turgidity and become flaccid. The leaves are next enveloped in filter-cloth and pressed in the Buchner press. The cells become flattened, but the cellulose wall is not broken, so that the aqueous liquid which is forced through it will contain only those water-soluble constituents of the cell whose molecules are sufficiently small to allow them to diffuse freely through the pores of the cellulose wall. The fatty materials, proteins, *etc.*, of the protoplasm, as well as any complex polysaccharides, proteins or proteoses present in the original vacuole fluid are retained inside the cell. The cell wall, in fact, is used as a very convenient ultrafilter. The press extract thus obtained is a clear brown fluid, while the residue inside the flattened cell is a deep green gel. On removal from the press the flattened leaves readily imbibe water and swell up to their original volume. By alternate pressing and soaking in water the simpler water-soluble constituents of the cells can be readily washed away. The leaf residues are next ground in a meat chopper with water. The leaf cells are now torn open, and the deep green gel of cytoplasmic material is dispersed into colloidal solution. The cell wall material is removed by squeezing through silk gauze and a green liquid is thus obtained which is heavily charged with colloidal protein and fatty material.

It is possible to separate this protein into two fractions by filtration through paper pulp. In former papers the fraction retained on the filter-pad, which cannot be readily separated from the fatty material, has been referred to as the "combined" protein, while that which passes freely through the filter has been referred to as the "soluble" protein. If the green colloidal solution is flocculated by heat the product will contain both the above-mentioned protein fractions. It will be referred to in the present and future papers as the "whole" protein of the leaf. Until more is known about the chemical and physical properties of the leaf-proteins such arbitrary distinctions are unfortunately necessary.

When applied to various samples of cocksfoot and ryegrass the above-mentioned ether method was unsatisfactory. The green colloidal solution obtained by grinding yielded very little colloidal protein, and on filtration through paper pulp it gave no "soluble" protein at all. It was clear that on cytolysis the leaf-proteins had undergone some form of denaturation similar to that which had already been observed in the case of *Cochlearia amoracea* [Chibnall and Grover, 1926]. Experiments were therefore made to find out in which way the procedure could be modified so as to prevent this apparent denaturation.

In previous researches the leaf material after cytolysis had been enveloped in filter-cloth and pressed between two flat iron plates in the Buchner press; in the present research it has been found more convenient to use a steel cylinder,

11 ins. high and of 6 ins. internal diameter, the pressure being applied by a well-fitting plunger. The "effective pressure" on the leaf material could in this way be made very much greater than when the former procedure was employed.

The cocksfoot used in the present research was obtained from the Agricultural Research Station of the Imperial Chemical Industries Ltd., at Jealott's Hill, Bracknell, Berks. About three weeks before a sample was required the plot was cut low with a scythe and heavily dressed with nitrogenous fertiliser. When the new growth stood 8 to 10 ins. high the upper 6 ins. of the blades of cocksfoot were removed with a scythe and used for the protein experiments.

Exp. 1. A sample of cocksfoot (Batch O), cut at 7 a.m. on September 17th, 1930. The fresh weight was 10 kg., the dry weight 13·5 % and the total N 3·5 % of the dry weight. As it was possible that the apparent denaturation of the protein which occurred in earlier grass experiments had been brought about by the ether it was decided to use ether-water as the cytolysing agent in the present case. The grass was accordingly immersed in ether-water for 20 minutes and then pressed for 10 minutes at 200–400 kg./cm.^{2} Three washings with water were given, and the green colloidal solution obtained after grinding the leaf residues was divided into two equal portions of 22·5 litres. One portion was filtered through paper pulp and the "soluble" protein flocculated at the isoelectric point by adding the requisite amount of mineral acid. It was then coagulated by heating on a water-bath to 95°, filtered, extracted twice with boiling water, again filtered, and then pressed to a hard cake in the Buchner press. The press-cake was powdered, extracted overnight with ether in a Soxhlet apparatus, and then extracted three times successively, first with boiling 95 % alcohol and finally with boiling absolute alcohol. The "soluble" protein, dried at 110°, weighed 25 g. and it contained 13·7 % of N and 2·7 % of ash. The N, ash-free, was 14·0 %. The other portion of the green colloidal solution was heated to 95° to coagulate the protein, which was then washed in the manner described above. The "whole" protein, dried at 110°, weighed 55·6 g., and it contained 11·4 % of N, and 7·7 % of ash. The N, ash-free, was 12·4 %.

Table I. *Comparison of the yields of "soluble" and "whole" proteins obtained from cocksfoot and spinach* [Chibnall, 1924].

	Percentage of total leaf-N		Percentage of total protein-N	
	Cocksfoot	Spinach	Cocksfoot	Spinach
"Whole" protein-N	28·8	30·9	34·3	42·6
"Soluble" protein-N	13·6	14·1	16·2	19·4
Water-soluble non-protein-N	15·9	27·6	—	—
Unextracted-N	55·3	40·5	65·7	57·4

The yields of the proteins are given in Table I, together with those from spinach obtained in an earlier experiment in which ether was used as the cytolysing agent [Chibnall, 1924]. The blades of cocksfoot are thin and fibrous,

and undergo much less disintegration in the meat chopper than fleshy leaves such as spinach. For this reason the yields of the grass proteins were considered satisfactory, even though about 65 % of the total protein remained in the cellular residues, presumably in cells that had not been torn open during the grinding.

Exp. 2. The pressing and washing of large amounts of leaf material are laborious, and the next experiment was planned to investigate in what way the yield of "soluble" protein was influenced by the magnitude of the pressure and the time during which it was applied.

2 kg. of cocksfoot (Batch S), cut at 7 a.m. on October 17th, 1930, were cytolysed about 3 hours later with ether-water and pressed for 20 minutes at the maximum pressure (500 kg./cm.^{2}). The hard press-cake was allowed to imbibe water for 10 minutes and again pressed at the maximum pressure for 20 minutes. The washing and pressing were repeated 4 times in all. On grinding the final press-cake with water very little material passed into colloidal solution, and the yield of "soluble" protein was negligible.

About 4 hours later another 2 kg. sample of the same batch of grass was treated in the same way, except that only one washing was given. The yield of "soluble" protein was 6 % of the total leaf-N. The preparation contained 11·7 % of N and 4·4 % of ash. The N, ash-free, was 12·3 %. Both the yield and purity of the protein were poor, but the experiment demonstrated that the pressing should not be too severe, and that the time of pressing and washing should not be unduly prolonged.

Attempts to increase the purity of the preparation by redissolving an aliquot of the freshly flocculated "soluble" protein and filtering the solution a second time through paper pulp were unsuccessful.

Exp. 3. Further supplies of pure strain grass were not available until late in the following spring. Batch E was cut at 7 a.m. on June 15th, 1931. The fresh weight was 12 kg., the dry weight 13·0 % and the N 3·2 % of the dry weight. The treatment applied was that which the earlier experiments suggested would be the most favourable for good yields of "soluble" protein.

A sample of grass weighing 2 kg. was cytolysed in ether-water for 10 minutes, and then pressed for 4 minutes at the maximum pressure. The residue was allowed to imbibe water for 4 minutes, and pressed as before. The washing was twice repeated. The final press-cake was ground with water and the "soluble" protein obtained in the usual way. The cellular residue was again minced with water to obtain a second green colloidal extract. Successive 2 kg. samples of grass were treated in a similar way at such time intervals that there was always sufficient green colloidal solution to permit of continuous filtration through the large paper pulp pad. The time required to carry through the treatment of each sample of grass to the filtration stage was only 55 minutes. The "soluble" protein from the 12 kg. of grass weighed 36·5 g., and it contained 12·6 % of N and 1·4 % of ash. The N, ash-free, was 12·8 %. The yield was 9·1 % of the total leaf-N.

It was noticed during this experiment that the amount of colloidal material in the green extracts increased with each successive sample of the grass, and that whereas the first gave a "soluble" protein filtrate which was coloured only a faint brown and contained very little protein, the fourth, fifth and sixth gave filtrates which were deeply coloured and contained a very large amount of protein. This observation suggested that some change affecting the "aggregation" of the colloidal leaf-proteins had taken place within a few hours of cutting, so that the sample treated after 3 hours gave much less "soluble" protein than that treated after 6–8 hours. Such a result was entirely unexpected, as it was contrary to that of previous researches in which ether had been used as the cytolysing agent [Chibnall and Nolan, 1924]. To obtain confirmatory evidence the following experiment was made.

Exp. 4. Batch F was cut from the same plot as Batch E at 7 a.m. on June 27th, 1931. The dry weight of the grass had increased to 16 %, due, presumably, to active photosynthesis during this period. Three samples of 2 kg. each were treated at $3\frac{1}{2}$, 7 and 10 hours respectively after cutting. The "soluble" proteins contained (ash-free) 12·4 %, 12·6 % and 12·6 % of N and the yields were 2·4 %, 7·4 % and 4·7 % of the total leaf-N respectively. This experiment showed quite clearly that the optimum working time for the best yield of protein was about 8 hours after cutting.

No further supplies of pure strain grass were available until September, 1931. With the experience gained in the above-mentioned experiments it was then confidently hoped that samples of about 500 g. of both "whole" and "soluble" proteins of cocksfoot could be prepared for amino-acid analysis. The expectation was not realised as the yield of protein from every sample treated was negligible. At the present time we are quite unable to explain this curious difference in behaviour. Many of the samples taken were from the identical 2-year old grass beds which had yielded abundant protein in the spring of 1931 and in September and October of 1930. Others were from 1-year old beds which had not before been used for protein experiments. To obtain samples of grass rich in protein all the beds had been heavily fertilised with either calcium nitrate or ammonium sulphate, and as the summer of 1931 was abnormally cloudy it is possible that some derangement of the normal carbon-nitrogen balance, or of the distribution of inorganic nutrients in the leaf, had affected the "aggregation" of the leaf-proteins in such a way that after cytolysis they were not readily dispersed into colloidal solution by grinding with water.

Analysis of the "soluble" proteins.

Purity of the various preparations. The ash and N analyses are collected in Table II. The N content is not constant throughout, and in most cases is disappointingly low, showing that the preparations contained 15–25 % of N-free material. A similar impurity had been encountered in a previous research [Chibnall and Grover, 1926] and, as in the present case, the amount present decreased with increase in the yield of "soluble" protein, suggesting that the

N-free material was not necessarily a prosthetic group in the protein molecule, but was some constituent of the leaf cell which had similar solubility properties to the "soluble" proteins.

Table II. *Showing details of "soluble" proteins prepared from cocksfoot.*

Preparation	N (%)	Ash (%)	Ash-free N (%)	Yield (% of total leaf-N)
O	13·6	2·7	14·0	13·6
S	11·7	4·4	12·3	6·0
E	12·6	1·4	12·8	9·1
F_1	12·2	1·8	12·4	2·7
F_2	12·4	1·7	12·6	7·4
F_3	12·4	1·3	12·6	4·7
G	12·5	5·1	13·2	2·4

The nature of this impurity has been investigated in some detail. All the protein preparations gave only 1% of furfuraldehyde on distillation with hydrochloric acid, showing that the impurity was not a pentosan or polyuronide such as pectin, both of which might well have been adsorbed on the protein when it was first flocculated from solution by acid. As all the preparations gave a strong Molisch test for carbohydrate the amount of reducing sugar yielded on mild acid hydrolysis was next investigated.

Estimation of reducing sugars after acid hydrolysis. The protein (1 g.) was hydrolysed with either 4 % or 10 % sulphuric acid for various periods. The hydrolysate was filtered, neutralised to Congo red with sodium carbonate and an aliquot used for determining reducing sugar. In some of the preliminary experiments Hanes's [1929] modification of the Hagedorn and Jensen method was employed. The results varied between 7 % and 10 % of the protein, and suggested that the impurity was a polysaccharide. It was then found that crystalline edestin when submitted to similar treatment gave 4·1 % of "reducing sugar," showing that the ferricyanide must be reacting with the hydrolysis products of the protein, and that therefore the method was inapplicable to the determination of reducing sugars in crude protein preparations. The method of MacLean [1919] gave lower, and what appeared to be more reliable results. The value for edestin was 1·4 %, and was clearly not due to any

Table III. *Showing the amount of reducing sugar obtained from the "soluble" proteins by acid hydrolysis.*

			Number of hours hydrolysis			
Preparation	N (ash-free) (%)	Strength of acid (%)	1 Reducing sugar (%)	2 Reducing sugar (%)	4 Reducing sugar (%)	16 Reducing sugar (%)
O	14·0	4	—	—	—	3·0
O	14·0	10	—	—	—	3·1
S	12·3	4	—	—	—	4·6
E	12·6	4	2·9	3·3	2·9	3·2
E	12·6	10	4·2	4·4	3·7	3·0
Edestin	18·5	10	—	1·4	—	1·4

reducing sugar, because a yellowish-white turbidity instead of a precipitate of red cuprous oxide was formed during the boiling with the copper reagent. In the case of the "soluble" leaf-proteins the reduction proceeded normally, and the values for reducing sugar given after various periods of hydrolysis are recorded in Table III. Assuming that this reducing sugar was derived from a polysaccharide the amount of the latter was clearly insufficient to account for all the N-free material present in the protein preparations unless on acid hydrolysis non-reducing substances were produced as well.

Estimation of polysaccharides by alkaline hydrolysis. To determine the optimum conditions of hydrolysis the following experiments were made. "Soluble" protein E (0·5 g.) was hydrolysed with 5 % or 10 % barium hydroxide ($Ba(OH)_2, 8H_2O$) (5 cc.) at 100° for various periods. The mixture was then filtered and the residue washed with hot water. The filtrate and washings were made just acid to Congo red with sulphuric acid, the precipitated barium sulphate was filtered off, and the clear liquid made up to 50 cc. Reducing sugars were determined by the method of MacLean both before and after hydrolysis with 0·7 % hydrochloric acid for 2 hours. The results are given in Table IV. Before the acid hydrolysis no appreciable amount of cuprous oxide

Table IV. *Estimation of polysaccharides in "soluble" protein E by alkaline hydrolysis.*

	After treatment with 5 % barium hydroxide for 2 hours at 100°		After treatment with 10 % barium hydroxide for 2 hours at 100°	
Time (hours)	Before hydrolysis. Sugar (%)	After hydrolysis. Increase in sugar (%)	Before hydrolysis. Sugar (%)	After hydrolysis. Increase in sugar (%)
0·5	—	—	0·56	2·02
1	0·25	2·07	0·23	2·34
2	0·25	2·33	0·36	2·08
4	0·31	2·41	—	—
7	—	—	0·85	1·62

was formed during the reduction stage, although subsequently there was a small titration value for "reducing sugar." After acid hydrolysis the reduction proceeded normally. It will be seen that the maximum yield of polysaccharide was obtained after hydrolysis with 5 % barium hydroxide for 2 hours. Several other "soluble" proteins from leaves, and also edestin, were then hydrolysed with 5 % barium hydroxide for 2 hours, and reducing sugars determined both before and after acid hydrolysis. The results are given in Table V. The edestin and the protein from spinach, both of which are free from carbohydrate (as shown by the Molisch test), gave a yellow turbidity instead of a precipitate of cuprous oxide during the reduction, and the titration value obtained must be due to the interaction of certain amino-acids with the MacLean reagents.

In the case of the proteins from *Zea Mays* and cocksfoot the reduction proceeded normally, suggesting that a small amount of polysaccharide had been set free during the alkaline hydrolysis. It was not found possible to estimate

the total polysaccharide in the leaf-proteins by the colorimetric method of Tillmans and Philippi [1929] because they had been coagulated and therefore were insoluble in dilute alkali.

Table V. *Estimation of polysaccharides in certain leaf-proteins and in edestin (after hydrolysis at 100° with 5 % barium hydroxide for 2 hours).*

Protein	N (ash-free) (%)	Increase in reducing sugar after hydrolysis with acid (%)
Grass "soluble" protein S	12·3	2·2
Grass "soluble" protein E	12·8	2·1
Grass "soluble" protein O	14·0	1·6
*Zea Mays** "soluble" protein	14·4	2·0
Spinach† "soluble" protein	16·2	1·0
Edestin	18·4	0·8

* Original sample prepared by Chibnall and Nolan [1924].
† Original sample prepared by Chibnall [1924].

Isolation of a small amount of polysaccharide. A collected sample of 50 g. of "soluble" protein was boiled for 2 hours under reflux (carbon dioxide excluded) in 500 cc. water containing 25 g. crystalline barium hydroxide. The hot mixture was filtered, the residue washed with hot water, and the filtrate and washings neutralised to litmus with sulphuric acid. The precipitated barium sulphate was centrifuged off, washed, and the clear liquid and washings were collected. The "reducing sugar" before hydrolysis with 0·7 % HCl was 0·16 g. and after hydrolysis 1·30 g. The increase was equivalent to 2·3 % of the original protein.

The remainder of the solution was treated with a saturated solution of lead acetate until precipitation was complete. The mixture could not be completely separated on the centrifuge, and it was necessary to treat the cloudy liquid with charcoal and filter. The clear filtrate was then treated with ammonia and lead acetate until precipitation was complete [Rimington, 1929]. The precipitate was centrifuged off, washed several times with a dilute solution of ammonia and then filtered under suction. The residue was redissolved by grinding with dilute acetic acid, a small amount of insoluble pigment removed by filtration, and the lead-sugar complex reprecipitated by adding ammonia. It was washed as before and then decomposed under pressure with hydrogen sulphide in the usual way. The precipitated lead sulphide was washed with hot water and the extract and washings were collected. The reducing sugar before hydrolysis with 0·7 % HCl was negligible, and after hydrolysis was 0·60 g., equivalent to 1·2 % of the original protein. To the remainder of the solution sufficient sulphuric acid was added to make the concentration 10 %, followed by 10 cc. of a 30 % solution of mercuric sulphate in 10 % sulphuric acid, to precipitate most of the nitrogen [Greenwald, 1930]. The mixture was cooled in ice and the sulphuric acid removed quantitatively by the addition of a hot saturated solution of barium hydroxide. The precipitate was removed by filtration and the filtrate

freed from mercury by means of hydrogen sulphide. The solution at this stage contained: total solids, 0·8 g.; total nitrogen, 0·009 g.; reducing sugar after hydrolysis, 0·3 g. It was next evaporated under reduced pressure to 15 cc., and the slight turbidity removed by filtration. To the filtrate were added 4 volumes of methyl alcohol followed by 4 volumes of ether and the solution was kept for 5 days. The precipitate, a white powder, was filtered off, washed with ethyl alcohol and kept for several days *in vacuo* over sulphuric acid.

The material (0·24 g.) had no definite M.P., but darkened slowly above 180°. (Found: ash, 11·5 %; ash-free, C, 43·9; H, 6·5 %. A polysaccharide requires C, 44·4; H, 6·1 %.) The following results are quoted ash-free: N, 1·2 %; $[\alpha]_D - 37{\cdot}0°$; reducing sugar after hydrolysis with 0·7 % hydrochloric acid, 48·3 %; total polysaccharide by the colorimetric method of Tillmans and Philippi [1929], 100 %. The small yield (1 %) and low nitrogen content of the polysaccharide, together with the relative ease with which it is set free from the protein on alkaline hydrolysis, did not suggest that it was an amino-trisaccharide of the type recently isolated by Levene and Mori [1929] from ovalbumin and ovomucoid, and by Rimington [1929] from both the albumin and globulin of horse's blood-serum. To obtain further evidence the following experiments were made.

50 mg. of the polysaccharide were hydrolysed with 0·7 % hydrochloric acid (5 cc.) for 2 hours on a boiling water-bath. The solution was neutralised with sodium acetate and treated with the requisite quantities of phenylhydrazine hydrochloride and sodium acetate, heating for 1 hour on a boiling water-bath. On cooling an orange-coloured crystalline precipitate with the appearance of an osazone separated out. This precipitate was filtered, washed and dried on porous tile, *in vacuo*, over sulphuric acid: M.P. 128–130°. There was not sufficient material for further characterisation.

Another 50 mg. were hydrolysed with 10 % sulphuric acid for 2 hours, the acid removed with barium hydroxide in the usual way and the clear solution treated with salicylaldehyde [Irvine and Earl, 1922]. No insoluble crystalline precipitate of salicylidene-glucosamine was formed, showing the probable absence of glucosamine.

From these results it seems probable that the polysaccharide is nitrogen-free, and that on acid hydrolysis it gives 50 % of reducing sugar.

The amount present (3–4 %) is only about one-fifth of the total nitrogen-free material which the soluble proteins must contain, and no further investigation has been made into its constitution.

Amino-acid analysis. Sample O, which contained (ash-free) 14 % N, was considered sufficiently pure for the usual Van Slyke analysis. The results are given in Table VI, together with those of certain other "soluble" leaf-proteins. Dicarboxylic acid-N was determined by the method of Damodaran [1931], some of whose results are also quoted. The analysis of the cocksfoot again illustrates the very close agreement in amino-acid composition which has been found in all the leaf-proteins so far analysed. The agreement is particularly

Table VI. *Van Slyke analyses of cocksfoot and certain other "soluble" leaf-proteins (in percentages of total nitrogen; nitrogen figures corrected for solubility of bases).*

	Cocksfoot	*Zea Mays**	Spinach†	*Medicago sativa**	Runner bean‡
Amide-N	5·9	7·4	6·9	5·5	5·7
Humin-N	6·5	4·6	2·5	2·7	4·2
Arginine-N	14·3	14·7	13·8	15·3	16·1
Histidine-N	5·3	4·7	3·9	3·1	6·2
Lysine N	8·3	8·8	9·6	10·0	10·1
Cystine-N	0·0	0·8	1·3	0·8	
Mono-amino-N	56·0	55·8	58·1	58·6	53·8
Non-amino-N	3·7	2·0	2·6	3·2	3·4
	99·7	98·8	98·7	99·2	99·5
Total basic-N	27·9	29·0	28·6	29·2	32·4
Dicarboxylic acid-N	19·9	21·9	17·7	20·1	20·8
% N in protein (ash-free)	14·0	14·4	16·25	15·8	15·85

* Chibnall and Nolan [1924]. † Chibnall [1924]. ‡ Chibnall and Grover [1926].

close in the case of *Zea Mays*, which also belongs to the Gramineae and contains about the same amount of N-free impurity.

In one respect however the cocksfoot protein differs from the others, and that is in the apparent absence of cystine. The sulphur content of the phosphotungstic acid precipitate was estimated in two ways. (i) After removal of phosphotungstic acid with amyl alcohol-ether and treating the bases with the Benedict-Denis reagent in the usual way [Van Slyke, 1915]. (ii) By the modified method of Plimmer and Lowndes [1927], in which the estimation was carried out without the previous removal of the phosphotungstic acid. This result is in harmony with that of Aitken [1930] who was unable to detect cystine in either cocksfoot or perennial ryegrass after hydrolysis of the whole grass with 20 % HCl.

The sulphur content of "soluble" protein E was 0·75 % and of O 1·0 %. Proof that the major part of the sulphur in preparation E was organic sulphur was obtained in the following way. The protein (1 g.) was hydrolysed with 20 % HCl (50 cc.) for 16 hours, excess acid removed by concentration *in vacuo*, and the acid humin filtered off and washed. The ash weight of the acid humin was 0·11 %. Assuming that this was all calcium sulphate the sulphur is equivalent to 0·03 %. The filtrate and washings were treated with barium chloride and HCl in the usual way. No precipitate of barium sulphate was obtained, showing the absence of inorganic sulphate. These results demonstrate that the major part of the sulphur in preparation E was organic sulphur, and unless this was derived from some N-free impurity, it follows that the protein must have contained a non-basic sulphur-containing amino-acid such as methionine. This interesting result will be investigated in greater detail when further supplies of grass proteins are available.

SUMMARY.

Various attempts have been made to prepare proteins in good yield from grasses. The original ether method of Chibnall was unsuccessful, but by substituting ether-water for ether good yields of protein have been obtained in some cases, but not in others.

The protein preparations have a low N-content (12·3—14 %), and contain varying amounts of N-free impurities which are difficult to remove. A polysaccharide is present in small amount, but the constitution of the major part of the impurity has not yet been determined.

A preparation containing 14 % of N has been analysed by the method of Van Slyke. The amino-acid composition was very similar to that of other leaf-proteins, but the presence of cystine could not be demonstrated, although the protein contained 1·0 % of sulphur.

Another preparation containing 0·75 % of sulphur gave only small amounts of inorganic sulphur, suggesting the presence in the protein of a non-basic sulphur-containing amino-acid such as methionine.

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XLVIII. WAX METABOLISM IN THE LEAVES OF BRUSSELS SPROUT[1].

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In connection with an investigation which is being made in this laboratory into the metabolism of paraffins in the higher plants it was thought that further light might be thrown on the chemistry of paraffin synthesis in the leaf if the composition of a leaf-wax known to contain large amounts of paraffin could be determined at various stages throughout the life history of the leaf. The wax of cabbage leaves (*Brassica oleracea* v. *capitata*), which was shown by Channon and Chibnall [1929] to consist chiefly of the paraffin *n*-nonacosane and the closely allied ketone 15-nonacosanone would have been a suitable one for such an experiment, but unfortunately the headed cabbage, which is a bud with its leaves densely compacted together and in different degrees of maturity, is unsuitable for this purpose. The Brussels sprout (*B. oleracea* v. *gemmifera*) on the contrary appeared to be quite suitable for this purpose and as the botanical difference between the two plants is a varietal one only it seemed to us probable that the leaf-waxes would be similar. Accordingly we first determined the composition of the Brussels sprout leaf-wax and having found that it consisted chiefly of *n*-nonacosane, 15-nonacosanone and 15-nonacosanol we investigated the change in composition throughout the life history of the leaf.

In the experiments detailed below it is to be clearly understood that the term Brussels sprout leaf refers to the leaves growing on the main axis of the plant, and is in no way concerned with the bud-like shoots commonly referred to as "sprouts."

Analysis of the wax.

Several samples of wax ("crude hydrocarbon") had been obtained in a previous research from the mature leaves of Brussels sprout by Chibnall and Sahai [1931]. These were collected and saponified and the unsaponifiable material (22 g.) was fractionated by the phthalate method of Chibnall *et al.* [1931].

Primary alcohols. Only a small amount of insoluble sodium salts of primary phthalates was obtained. This was thoroughly extracted with ether and then

[1] The melting-points recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

saponified in benzene-alcoholic sodium ethoxide. The recovered primary alcohol was crystallised from light petroleum; yield 0·8 g.; M.P. 79°. A collected sample from this and later experiments weighed 1·3 g., and from it the following derivatives were prepared by the methods used by Pollard *et al.* [1931]. Acetate, M.P. 60·7–61°; acid M.P. 83–83·4°; paraffin M.P. 59·7–60° with no definite transition temperatures, showing that it was a mixture of more than 3 paraffins of mean molecular weight corresponding to C_{27} [Piper *et al.* 1931]. The primary alcohol was undoubtedly a mixture of the type which we suggested in an earlier paper [Pollard *et al.* 1931] should be called "ceryl" alcohol.

Ketones. After the removal of the insoluble sodium salts of the primary phthalates the ethereal solution was evaporated to dryness and the residue dissolved in about 200 cc. of boiling alcohol. On cooling 14·5 g. of material, M.P. 70°, crystallised out. This was treated with phthalic anhydride (14 g.) at 120° for 18 hours, and a further small amount of primary phthalates removed as before. On again crystallising the residue from hot alcohol 11·7 g. of material were obtained. The two alcoholic mother-liquors were evaporated to dryness and gave the crude secondary phthalate fractions mentioned below.

The 11·7 g. of crystalline material were stirred at room temperature with 100 cc. of light petroleum (B.P. 40–60°) and the solvent filtered off. This operation was repeated until no more material appeared to go readily into the cold solvent. The residue was dissolved in 100 cc. of warm light petroleum. On cooling 1·98 g. of material crystallised out; M.P. 77·5°. This was recrystallised from alcohol (charcoal), then from light petroleum and finally from carbon disulphide. The yield was 1·1 g.; M.P. 80·6–81°, unchanged in a mixed melt with synthetic 15-nonacosanone (M.P. 80·8°). The oxime was made in the usual way; M.P. 52·5°, unchanged when mixed with synthetic oxime (M.P. 52–53°). (Found: N, 3·3. $C_{29}H_{58}NOH$ requires N, 3·2 %.)

The mother-liquor from the warm light petroleum crystallisation was taken to dryness and the residue repeatedly extracted with cold light petroleum (1 : 10) as before. The final residue was crystallised first from carbon disulphide and then from alcohol (charcoal). A further 0·72 g. of ketone M.P. 80·5° was thus obtained.

The mother-liquors from all the cold extractions with light petroleum were next combined and the solvent removed. The product (M.P. 63–64°) could not be further fractionated by mixed solvents, but as very considerable charring occurred on treatment with sulphuric acid at 120° it was surmised that ketone was still present. It was therefore oximated in the usual way, using 11 g. of hydroxylamine hydrochloride, 11 g. of potassium hydroxide and 200 cc. of alcohol. The mixture was boiled for 12 hours and filtered hot to remove potassium chloride. The filtrate was allowed to cool slowly, when 7·1 g. of practically pure paraffin crystallised out.

The alcoholic mother-liquor was evaporated to 150 cc. and 100 cc. of water were added. The white precipitate was filtered off; weight 1·45 g.; M.P. 50–51°. It was dissolved in a warm mixture of light petroleum (20 cc.) and acetone

(20 cc.) and allowed to cool. 0·1 g. of crude paraffin crystallised out and was filtered off. The mother-liquor was evaporated to dryness and the residue dissolved in warm acetic acid (50 cc.). No precipitate separated on cooling, showing that all the paraffin had been removed. The acetic acid solution was evaporated to 15 cc. and an equal volume of warm acetone added. On cooling 1·1 g. of ketoxime crystallised out; M.P. 51·5°, unchanged in a mixed melt with synthetic 15-nonacosanone oxime (M.P. 52–53°). 1 g. of the ketoxime was saponified for 12 hours in alcohol saturated with hydrogen chloride. 0·75 g. of 15-nonacosanone M.P. 80·6–80·9° was thus obtained. The total yield of ketone was therefore 2·6 g.

Secondary alcohol. The two fractions of the sodium salts of the secondary phthalates were united and saponified in benzene-alcoholic sodium ethoxide, and the secondary alcohol was extracted with hot acetone. The 1·6 g. of white crystalline material which separated on cooling was recrystallised from hot alcohol (charcoal); M.P. 82·3–82·8°. After 3 recrystallisations from pyridine the M.P. was raised to 83·5–83·8°. (Found: C, 81·9; H, 14·3. $C_{29}H_{60}O$ requires C, 82·0; H, 14·2 %.) The presence of *n*-nonacosane and 15-nonacosanone in the wax suggested that this secondary alcohol might be 15-nonacosanol, M.P. 83·6–83·8°, which had not hitherto been found in natural products, but which had been recently synthesised by Piper *et al.* [1931]. This was confirmed by a mixed melt, which showed no depression. The acetate of the natural alcohol was prepared in the usual way, M.P. alone, and mixed with the synthetic acetate, 50·5–51°.

Paraffin fraction. The 7·1 g. of paraffin obtained during the oximation mentioned above melted at 63–64°. On treatment with sulphuric acid at 120–130° very little charring occurred. After washing with water the product was dissolved in hot alcohol and filtered hot to remove traces of carbon. On cooling the paraffin (6·7 g.) crystallised out in small white leaflets. The melting-point 63·7–64° showed that it was a mixture. The material was accordingly fractionated by means of light petroleum [Chibnall *et al.* 1931]. Fractionation was slow, and after some 50 samples had been dealt with we obtained a series of 9 fractions differing slightly in M.P. The melting- and transition-points of the upper, lower and two representative intermediate fractions are given in Table I. Each fraction was melted alongside a sample of synthetic $C_{29}H_{60}$.

Table I. *Fractionation of the paraffin.*

No.	Heating transition-point	M.P.	S.P.	Cooling transition-point
1	57·0–57·3°	63·5–63·7°	63·3°	55·5°
2	56·5–57·0	63·7–63·9	63·5	55·5
3	56·5–57·0	63·9–64·1	63·6	55·4
4	56·3–56·7	64·1–64·3	63·9	55·2
$C_{29}H_{60}$	57·3–57·5	63·4–63·6	63·2	55·8

From the data for mixtures of $C_{29}H_{60} + C_{31}H_{64}$ and $C_{27}H_{56} + C_{28}H_{58}$ given in a previous paper [Piper *et al.* 1931] we conclude that the Brussels sprout paraffin

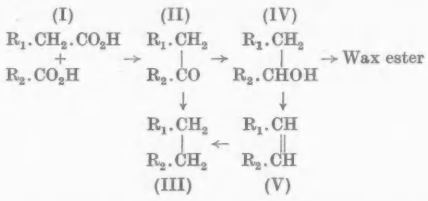
fractions consist of $C_{29}H_{60}$ with 2·5 to 10 % of $C_{31}H_{64}$. If the second paraffin had been $C_{30}H_{62}$ we should not have expected in the case of fraction 4 a fall of nearly 1° in the heating transition-point.

Fatty acids. The small amount of fatty acid recovered from the soaps after saponification was deep green, and required repeated treatment with charcoal in boiling alcohol to obtain a white crystalline product. The M.P. was 79–79·5° showing that the acid was the usual "cerotic" acid of plant waxes.

The constituents of the wax.

It will be seen that the chief constituents of the wax are the three closely allied substances *n*-nonacosane, 15-nonacosanone and 15-nonacosanol. In addition small amounts of mixed higher primary alcohols (ceryl alcohol), mixed higher *n*-fatty acids (cerotic acid) and another paraffin, probably *n*-hentriacontane are present.

In a previous paper [Chibnall *et al.* 1931] it was shown that the chief constituents of apple-peel wax were *n*-nonacosane and *d*-10-nonacosanol. Although the corresponding ketone 10-nonacosanone was not found it was suggested that the original hypothesis of Channon and Chibnall [1929] for the synthesis of higher paraffins in the plant might be extended in the following way:



The presence in the same wax of a paraffin (III) and both its corresponding ketone (II) and secondary alcohol (IV) certainly lends additional support to such an inter-relationship. It was pointed out however that if the ketone (II) arises through the condensation of the molecules of fatty acid (I), then 15-nonacosanone would postulate the presence in the plant at some stage in the synthesis of pentadecoic acid, and, as is well known the presence of this acid in natural products has been repeatedly disproved.

This weighty objection induced us to carry out two further experiments with Brussels sprout leaves.

A. An attempt has been made to demonstrate the presence of the olefin 14-nonacosene, $CH_3.(CH_2)_{12}.CH:CH.(CH_2)_{13}.CH_3$.

B. The composition of the wax has been investigated at three periods in the life history of the leaf. If either the ketone (II) or the secondary alcohol (IV) should be the precursor of the other two products, then it might be expected that the wax of the young leaves would contain relatively more of this substance than the mature or the aged leaves.

Attempted isolation of an olefin.

14-Nonacosene and certain other olefins have been synthesised in this laboratory by Dr Pollard from symmetrical secondary alcohols by the method of Grün *et al.* [1926]. These authors showed that both *cis*- and *trans*-isomerides were produced, one of which had a M.P. 20–30° higher than the other. The higher-melting isomerides crystallise in flakes similar to the corresponding paraffins. They are very soluble in cold light petroleum and are precipitated on the addition of 2 volumes of acetone. The lower-melting isomerides are even more soluble in cold light petroleum, but are not precipitated on the addition of acetone. These solubility relationships suggest that if a higher-melting isomeride were present in the ether extract of Brussels sprout leaves it would be precipitated by acetone with the wax fraction, whereas if the lower-melting—more soluble—isomeride were present it would not be precipitated at this stage, and would pass ultimately into the unsaponifiable fraction of the ether-acetone filtrate.

In the search for the higher-melting isomeride in the wax fraction it was necessary to attempt isolation before treatment with phthalic anhydride, as this reagent might give rise to an olefin by dehydration of the secondary alcohol known to be present. Accordingly the crude unsaponified wax (9·4 g.) was dissolved in 10 volumes of warm light petroleum (B.P. $< 40°$), which was then poured into a dish and left exposed to the air. On evaporation of the solvent the crude wax was left as a fine friable powder. This was then stirred at 15° with 100 cc. of light petroleum for 2 minutes, and the solvent filtered off. The operation was repeated. The combined filtrates were evaporated to dryness, and the residue was taken up in hot alcohol (20 cc.). The material that crystallised out on cooling (0·54 g.) should have contained the major part of the olefin present in the crude wax. The iodine value was 2 and M.P. 63·5–64°. As 14-nonacosene has an iodine value of 62·7 it is clear that the crude wax contained no appreciable amount of olefin.

Dr Pollard is investigating the possible presence of olefins in various leaves, and we have to thank him for permission to publish a brief account of his results showing that the unsaponifiable fraction of the ether-acetone filtrate of Brussels sprout leaves contains no lower-melting isomeride of 14-nonacosene. The unsaponifiable material (15 g.) was treated successively with light petroleum, ethyl alcohol and acetone. 3 g. of crude sterol and 2 g. of residual wax material were separated. The oily residue (10 g.) was dissolved in ethyl acetate (200 cc.) and hydrogenated with hydrogen and palladium in the presence of activated charcoal. The procedure employed readily reduced synthetic olefins to the corresponding paraffins. Although 500 cc. of hydrogen were absorbed the resulting product was still freely soluble in cold acetone, showing that no paraffin had been produced by reduction of an olefin.

The above results show fairly conclusively that no appreciable amount of olefin can have been present in the Brussels sprout leaves.

Changes in the composition of the wax during the life history of the leaf.

Materials used. The seeds which were to provide the plant material for samples 1–3 were sown in open beds on April 17th, 1931. At the same time seeds were sown in boxes which were kept in the open air alongside the beds. Late in May the seedlings growing in these boxes were transplanted into the same open beds, and at a later date provided the material for samples 4–6. The seedlings appeared above the ground about 5–7 days after sowing.

Details of the samples collected are given in Table II. Those of stages 1 and 2 consisted of seedlings which were cut just below the cotyledons, so that

Table II. *Showing the amount of wax in leaves of various ages.*

Stage	Age above ground in days	Condition of plants	Fresh wt. kg.	Dry wt. %	% of fresh wt.			% of dry wt.		
					Total ether extract	Wax	Glyceride fatty acids	Total ether extract	Wax	Glyceride fatty acids
	—	Ungerminated seed	0·1	—	37·8	0·0	33·8	—	—	—
1	10	Seedlings 2 cm. high, consisting of hypocotyl and cotyledon leaves	0·92	12·3	0·64	0·05	0·17	5·2	0·42	1·4
2	22	Seedlings 3–5 cm. high, 1st plumular leaf developing	13·1	11·8	0·83	0·10	0·16	7·1	0·85	1·4
3	43	Young plants 8–10 cm. high with 2–4 plumular leaves	0·92	11·8	0·74	0·13	0·15	6·3	1·1	1·3
4	54	Growing plant, laminae 8–10 cm. long	1·6	11·9	0·75	0·14	0·14	6·3	1·2	1·2
5	126	Mature plants, laminae 15–25 cm. long	22·0	15·7	0·49	0·18	0·09	3·2	1·2	0·5
6	221	Aged plants, laminae beginning to show signs of chlorophyll generation	12·0	Not taken	—	0·21	0·11	—	—	—

the material for analysis included the young plumule, but not the hypocotyl. Those of stages 3–6 consisted of detached laminae without petioles.

Preparation of the wax. To ascertain if the seeds contained any appreciable amount of wax 100 g. were powdered in a coffee mill and then extracted with ether in a Soxhlet apparatus for 24 hours. The ether extract was pale yellow, and on removal of the solvent weighed 37·8 g. 23·5 g. of this material were saponified in the usual way and gave 21 g. of fatty acids of iodine value 112 and 0·63 g. of unsaponifiable material. The latter was dissolved in 25 cc. of acetone and kept at 0° for some days. A small amount of phytosterol, M.P. 134°, crystallised out, but no constituent of the leaf wax.

The fresh leaf material was treated by the method of Chibnall and Sahai [1931] to obtain the ether extract, from which the wax was prepared in the usual way. It will be shown in a later communication that the percentage of

ether-soluble material obtained from leaves previously dried and ground to a powder is sometimes higher than that obtained from fresh material treated by the method quoted above. According to our limited experience this is only the case with leaves having a high surface/weight ratio such as grasses or runner bean, which dry very rapidly ($\frac{1}{2}$–1 hour) in an oven at 100°. Fleshy leaves such as the laminae of Brussels sprout, which do not dry rapidly in an oven, and which are fairly completely disintegrated in a meat chopper are best treated by the method quoted above.

It will be seen from Table II that the percentage of wax in the fresh leaf increased steadily with growth, showing that synthesis of wax must have gone on continuously throughout the life history of the leaf. To ascertain if this accumulation of wax had been accompanied by a change in composition, as it might have been, for instance, if paraffins had been formed continuously from ketones or secondary alcohols, the samples of waxes prepared from young (stage 2), mature (stage 5) and old (stage 6) leaves, which weighed 10·9 g., 40 g. and 25·5 g. respectively, were submitted to complete analysis by the method given below.

Analysis of the wax of young, mature and old leaves. In the analysis of the wax described earlier in this paper the ketone was separated from the paraffin partly by fractional crystallisation and partly by oximation. This procedure was laborious, and much material was unavoidably lost. To expedite the procedure the following modification was employed in the present instance. The primary and secondary alcohols were first removed as sodium phthalates in the usual way. The mixture of ketone and paraffin was then reduced with sodium in alcohol [Kipping, 1890], and the resulting secondary alcohol removed as sodium phthalate. The procedure in the case of the wax from the young leaves (stage 2) will illustrate the modified method employed.

The wax (10·9 g.) was saponified in benzene-alcoholic potassium hydroxide for 2 hours, and gave 0·6 g. of fatty acid and 9·0 g. of unsaponifiable material. The latter was treated twice successively with phthalic anhydride in the usual way. 0·74 g. of primary phthalate was obtained, which yielded on saponification 0·22 g. of primary alcohol. The combined secondary phthalates weighed 2·5 g. and gave 1·1 g. of secondary alcohol, M.P. 82·5°. The mixture of ketone and paraffin (5·9 g.) was dissolved in boiling alcohol (250 cc.) and sodium (20 g.) added in small portions at a time during 2 hours. On cooling the reduced ketone and paraffin crystallised out and were removed by filtration. This

Table III. *Analysis of the wax of young, mature and old laminae.*

	Young lamina (Stage 2) %	Mature lamina (Stage 5) %	Old lamina (Stage 6) %
Higher fatty acids	5·4	6·2	7·9
Primary alcohol	2·0	1·3	3·2
15-Nonacosanol	9·5	6·9	11·4
15-Nonacosanone	12·2	11·5	9·9
n-Nonacosane	32·9	26·0	25·1
Total accounted for	62·0	51·9	57·5

material (5·8 g.) was then treated twice successively with phthalic anhydride. 3·57 g. of paraffin and 2·3 g. of secondary phthalate, giving 1·49 g. of secondary alcohol (M.P. 83°), were obtained.

The results of the three analyses are given in Table III. The losses are considerable, due in large part to the nature and number of the operations through which the material is passed. They should, however, be of the same order in each case, so that it seems justifiable to conclude that the composition of the wax has not materially altered throughout the life history of the leaf.

Discussion.

The two well-known leaf-waxes of commerce, candelilla wax from *Euphorbia antisyphilitica* and carnäuba wax from *Copernicia cerifera* occur as epidermal excretions covering all parts of the plant except the roots. By analogy it has generally been assumed that all other leaf-waxes, most of which have hitherto been obtained only in small amounts, are also of epidermal origin, and that their main function in the plant is to reduce transpiration to a minimum.

Chibnall and Channon [1929] have already called attention to the fact that the wax of cabbage leaves, which may amount to 1 % of the dry weight, must be considered an integral part of the cytoplasmic material of the cell, because the leaves were treated in such a way that the wax was obtained from an ether extract of coagulated cell contents which was free from cell-wall material. It can be shown in a similar way that the wax of Brussels sprout leaves is also a constituent of the cell cytoplasm. It follows therefore that the rôle which these two latter waxes play in the physiology of the leaf cannot be directly concerned with transpiration, but must be connected in some way with the normal metabolism of the cells of the mesophyll.

Table IV. *Showing the amount of wax in various parts of seedlings of Brussels sprout.*

		g. per kg. fresh material		
		Dry weight	Ether extract	Wax
Stage 1.	Cotyledon leaves	90	5·7	0·28
	Hypocotyl	90	3·3	0·30
Stage 2.	Leaves	120	8·3	1·0
	Hypocotyl and rootlets	131	4·4	0·4

It is not surprising therefore that we have been unable to demonstrate the presence of wax in the fatty reserve of the seed cotyledons. But at an early stage of germination rapid synthesis of wax must have taken place, for at stage 1 (Table IV), when the young embryos consisted of a short hypocotyl and two rudimentary cotyledon leaves, the amount present in all parts of the embryo was considerable. In subsequent development of the plant the growth of the leaves was accompanied by a slow increase in the amount of wax, as

shown in Table II. If wax synthesis had been confined to new, actively growing, cells the amount present, expressed as a percentage of the fresh weight of the leaf, would have remained constant. Actually there was a definite increase throughout the life history of the leaf, showing that synthesis must have gone on continuously in some, at least, of the fully-grown cells. This slow accumulation suggests that the wax may either play some vital part in the physiological activity of the cell or be an essential metabolic by-product which is innocuous and chemically so inert that it is not further metabolised. The chemical evidence supports this latter suggestion.

Reverting to the suggested inter-relation between the constituents of the wax given on p. 406 it will be seen from the data given in Table III that no *ad hoc.* evidence has been obtained, because the composition of the wax has remained unchanged throughout the life history of the leaf. There is no accumulation of paraffin or secondary alcohol at the expense of the ketone, nor can the total increase in the wax constituents be accounted for by the small fall in glyceride fatty acids (Table II), of which only about 10 % are mixed saturated acids.

If the ketone is indeed the precursor of the paraffin and secondary alcohol, then it is possible that there is an equilibrium between these substances which is not disturbed by changes in the metabolic activity of the cell which accompany growth and senescence. It seems to us, on the contrary, more reasonable to assume that all the wax components, and not only the paraffin, are end-products of metabolism. Such a view does not exclude the possibility that the paraffin, ketone or secondary alcohol may have a common origin at some stage in the plant metabolism. We shall defer further discussion until our search for intermediary metabolites is more complete.

Summary.

The following constituents of Brussels sprout leaf-wax have been definitely identified. *n*-Nonacosane, 15-nonacosanone, 15-nonacosanol, ceryl alcohol, cerotic acid and probably *n*-hentriacontane. Olefins are absent.

As the wax contains a high proportion of paraffin an attempt has been made to throw light on paraffin metabolism by investigating the composition of the wax at various stages throughout the life history of the leaf.

The seeds contained no wax, but synthesis took place rapidly in all parts of the embryo, and went on continuously in the leaf throughout the life history of the plant. The composition of the wax did not alter throughout this period, and no evidence was obtained to support the hypothesis that the paraffin had been formed by reduction of the ketone. It is suggested that all the components of the wax are end-products of metabolism.

We should like to thank Mr Hales, of the Chelsea Physic Garden, for the care bestowed on the plants used.

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XLIX. THE ASSAY OF TESTICULAR HORMONE PREPARATIONS.

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Up to the present time the following tests have been used for the assay of testicular hormone.

The comb growth of capons is the test which has been most used [Dodds *et al.*, 1930; Freud, 1930; Funk and Harrow, 1929, 1930; Koch and Gallagher, 1929; Gallagher and Koch, 1930; Kabak, 1931; McGee, 1927; McGee, Juhn and Domm, 1928; Pezard, 1918]. This test, the details of which were worked out by Gallagher and Koch [1930] has many advantages.

(i) Only a very short time (5 days) is claimed to be necessary for its completion.

(ii) The results are accurately expressed in figures of increase of length and height of comb.

(iii) The same birds can be used for several assays, since after cessation of the injections the comb decreases again to its previous size.

The test has the following disadvantages.

(i) The comb, although differing in size and appearance, is an organ present in both sexes, and comb growth could, therefore, be obtained in birds of both sexes after injection of the hormone [Freud, 1930; Champy, 1931]. Injections of yolk and of ovarian extract give similar comb growth to that produced by testicular extract [Champy, 1931]. Therefore the hormone producing comb growth is considered by Champy to be specific for both sexes.

(ii) Sometimes an extract which is inactive on comb growth is active on growth of genital organs of castrated guinea-pigs and *vice versa* [Champy, 1931].

(iii) High doses of testicular preparations give exceedingly irregular results [Gallagher and Koch, 1930].

(iv) Although small doses give satisfactory results in assays, considerable variations are obtained, as is shown by the following experiments of Gallagher and Koch [1930, p. 318]. Forty-five capons were injected with the same dose of the same testicular preparation. In 2·2 % of them no comb growth was obtained; in 13·3 % 2 mm., in 15·5 % 3 mm., in 28·8 % 4 mm., in 28·8 % 5 mm., in 4·4 % 6 mm., and in 6·6 % 7 mm. increase in comb growth was obtained.

Still more striking variations were obtained by Kabak [1931], who, for example, on injecting 6 capons for 10 days, obtained the following increases in comb growth, 23, 13, 11, 9, 6, and 6 mm.

Tests on mammals. The following tests have been proposed.

On rats.

1. Sperm motility test [Moore, 1927, 1928].
2. Prostate cytology [Moore and Gallagher, 1929; Moore, Price and Gallagher, 1930].
3. Seminal vesicle cytology [Moore and Gallagher, 1929].
4. Vas deferens cytology [Vatna, 1930].
5. Cowper's gland cytology [Hiller, 1930].

On mice.

6. Seminal vesicle cytology (Loewe and Voss, 1930].

On guinea-pigs.

7. Electric ejaculation test [Moore and Gallagher, 1929].

All these tests are satisfactory, but Gallagher and Koch have objected to them, because either they take too long or lack quantitative precision in the end-point reading, or have both deficiencies. The necessity of operating on new animals for each assay is also a disadvantage in nearly all these tests.

Measurement of the size of the prostate, or of seminal vesicles, or of both, has also been used [Korenchevsky, 1928; Korenchevsky and Schultess-Young, 1928; Freud, 1930; Dodds *et al.* 1930]. This method, however, was not worked out as it was found by some authors (Freud, Dodds) to give contradictory results.

Weighing of prostate with seminal vesicles and of penis. I have, in previous publications [Korenchevsky, 1928, 1930; Korenchevsky and Schultess-Young, 1928], besides lineal measurements, used as a test of the activity of the extracts or for other purposes the increase in weight of the prostate with seminal vesicles, and of the penis of castrated rats. The aim of this paper is to give an accurate description of the method as it is now used.

The present investigation.

The lineal measurements of the sexual and other organs were found in our experiments to be unsuitable, since (1) it is not easy to make accurate measurements of organs of irregular shape and (2) the results have to be expressed in at least two figures, the length and width or height of the organ.

I have, therefore, chosen the weight of the organs as a criterion giving only one figure, and as being at the same time the most precise in end-point reading and a measurement of organs irrespective of their shape. Certain precautions, however, have to be taken to obtain accurate results. Some of these precautions have already been mentioned elsewhere [Korenchevsky, 1930].

1. *Feeding the animals.*

It is necessary, unless it is otherwise required, to feed all the animals used for the test on the same diet, which must be adequate in every respect. A synthetic diet in the form of a paste is used of the following composition:

Caseinogen	18	Dried yeast	15
Starch	50	Lemon juice	5
Butter	15	Water	64
Salt mixture	5		

In addition to this diet the animals receive daily 1 g. of wheat germ and 3 to 6 drops of cod-liver oil, for it is well known that for good development of the sexual organs nearly all the vitamins are of the greatest importance. A daily record of the amount of paste consumed by each rat is made. The object of giving this diet is first to supply the greatly altered organs of the castrated animals with all the factors necessary for a return to the normal condition, and, in addition, to measure the appetite of the injected animals, since testicular preparations frequently contain substances which depress the general functions and the appetite. If the appetite is decreased, the satisfactory regeneration of the atrophied organs of the castrated animals is difficult. Further, our experience has shown us that the decrease in appetite is one of the best and easiest tests for the demonstration of toxic depressing substances present in the testicular preparation, resulting from insufficient purification.

2. *Measurement of changes produced in organs.*

In castrated animals the following changes are observed [Korenchevsky, 1930]: atrophy of the prostate, seminal vesicles, penis and thyroids, hypertrophy of the adrenals and hypophysis, and increase in body fat. Under the influence of injections of testicular preparations, containing all the testicular hormones, these changes should return towards normal or even disappear.

Rats for examination are killed by coal gas. A quick and simple method for the accurate determination of fat deposition in the body of rats by means of the estimation of the weight of retroperitoneal fat has already been described [Korenchevsky, 1930]. The penis, after separation from the skin, is cut off at the base, where it is attached to the pelvic bones, and weighed, as is also the fat, without further treatment. Until weighed the fat and penis are kept in large covered crystallising dishes on two thicknesses of filter-paper moistened with saline solution. If the weighing has to be postponed the fat and penis can be kept in the dish in the cold store for several days without influencing the accuracy of the results. All the other organs are accurately but quickly separated from the body and immediately plunged for fixation and preservation in Allen's modification of Bouin's fixative. This contains saturated picric acid 75 cc., formalin 15 cc., glacial acetic acid 10 cc., urea 1 g. The seminal vesicles are always dissected as attached to prostate and the two weighed together, otherwise the secretion of the seminal vesicles would be lost. If necessary,

beside the organs mentioned above, pieces of other tissues are also cut off and plunged into the fixative.

Bouin's fixative, unlike others, does not change the weight of the organs appreciably, and in any case gives satisfactory results for comparative investigations.

After fixation is completed (2–3 days) the organs of the rats are placed in a large crystallising dish on two thicknesses of filter-paper moistened with saline solution, the organs of each rat being covered with a small Petri dish in order to prevent evaporation and consequent drying of the glands. It is possible in this way to place in one crystallising dish the organs of 7 rats, without any fear of mixing them. To prevent evaporation of the water from the filter-paper on which the 7 groups of organs are placed, the large dish in its turn is covered with a lid, which is only removed to take out or replace the gland. The weighing is made in a very small weighing-bottle on a chemical balance.

The treatment of the glands with Bouin's fixative was introduced for the following reasons: (1) the fixed glands are not affected so readily as fresh glands when exposed to the air or when kept, nor is their weight so easily changed; (2) after weighing, the organs can be used for histological investigation whilst, without fixation, artificial changes are produced during the somewhat lengthy process of dissection from the rat and subsequent weighing; (3) when weighed they do not stick to and do not soil the weighing-bottle, as do fresh glands; (4) it is very much easier to make the final separation of the fat from very atrophied prostate and seminal vesicles, if the organs are fixed. This is a matter of some importance since the prostate and seminal vesicles are the chief organs used in the assay.

3. *Fluctuations in weight of organs of non-injected castrated rats.*

Before considering the changes in weight of the prostate with seminal vesicles, penis, thyroids, adrenals, hypophysis and retroperitoneal fat of castrated rats as tests of the influence of testicular injections it is necessary to ascertain what are the normal fluctuations in weight of these organs in non-injected castrated rats.

For this purpose I have used 25 pairs of castrated rats, each pair having served for different experiments as the controls to their litter-mates injected with different preparations. The weights of the organs were calculated per 200 g. of rat weight. In each pair the average weight of each organ was found and the individual deviation from this average calculated as a percentage for each pair. For example, in litter 1497, the weights of prostate with seminal vesicles in the two rats were 0·208 g. and 0·195 g. Average weight 0·2015 g. Deviation from the average $\pm$ 0·0065 g. or $\pm$ 3·2 %. These deviations expressed as % for each organ for each pair are collected in Table I, arranged in ascending order of magnitude.

The difference in weight of the organs of the two rats of the pair was also calculated in a different manner. Taking the same example as mentioned above,

Table I. *Percentage deviations of weight of organs from their average weight in each pair of castrated uninjected rats of the same litter.*

Prostate with seminal vesicles	1·1, 1·8, 2·1, 3·2, 3·6, 3·6, 4·0, 4·1, 4·2, 5·1, 5·1, 5·9, 6·1, 6·3, 6·6, 7·2, 7·7, 8·9, 9·4, 9·4, 10·3, 10·9, 13·2, 13·3, 16·8 Average = **6·8**
Penis	0, 1·5, 2·1, 2·5, 2·8, 3·7, 4·8, 5·0, 5·6, 5·8, 8·6, 8·8, 10·1, 10·2, 10·6, 12·0, 12·0, 12·3, 12·4, 12·4, 13·4, 13·8, 16·1, 16·6, 17·7 Average = **8·8**
Adrenals	0·4, 0·8, 1·7, 2·1, 3·2, 3·7, 3·8, 4·2, 4·2, 4·7, 4·7, 6·0, 6·4, 8·1, 12·9, 13·0, 13·2, 15·4, 16·1, 16·7, 17·1, 17·4, 17·4, 23·3, 26·9 Average = **9·7**
Thyroid	0, 0·5, 0·5, 2·0, 2·3, 2·7, 3·6, 3·9, 4·1, 4·3, 4·8, 5·0, 6·0, 6·8, 7·7, 8·6, 9·7, 11·4, 14·1, 15·2, 15·7, 17·7, 17·9, 18·0 Average = **7·6**
Hypophysis	0, 1·2, 1·4, 2·3, 2·6, 2·7, 3·5, 3·8, 5·3, 5·4, 5·7, 6·7, 7·4, 7·5, 7·6, 8·4, 8·5, 8·7, 9·9, 10·6, 15·5, 18·5, 20·7 Average = **6·9**
Retroperitoneal fat	0·1, 0·3, 1·5, 3·5, 3·9, 4·8, 4·9, 4·9, 5·7, 7·1, 9·2, 9·5, 10·7, 10·9, 12·0, 12·1, 14·0, 21·2, 21·8, 25·8, 29·0, 29·8, 35·0, 37·0, 59·0 Average = **14·9**

litter 1497, the difference in weight of the prostate with seminal vesicles of the two rats is 0·013 g. (0·208 g–0·195 g.) which is 6·7 % of 0·195. In these calculations the percentage was taken with reference to the lower of the two weights referred to, the aim being to obtain the greatest difference of the figures. From the percentage difference in weight of the organs of the two rats of a pair, an average figure was obtained for each gland. All these average figures and the maximum and minimum percentage differences are given in Table II.

Table II. *Maximum, minimum and average percentage differences in weight of organs in each pair of uninjected castrated rats.*

Organs	Average difference	Maximum difference	Minimum difference
Prostate with seminal vesicles	15·3	34·3	3·6
Penis	19·1	41·8	0·0
Adrenals	22·3	73·7	1·7
Thyroid	17·1	43·5	0·0
Hypophysis	19·2	88·9	1·2
Retroperitoneal fat	29·7	117·2	0·2

To economise space the detailed figures are not given since the percentage figures of the differences of the weights of the glands of two rats can be roughly estimated as a little more than twice the respective figures given in Table I.

Examination of the figures given in Tables I and II shows that the weights of the prostate with seminal vesicles, of the penis and of the thyroid of castrated rats belonging to the same litter do not vary very considerably. The

maximum percentage deviation from the average weight of these respective organs in two litter-mate rats was 16·8, 17·7 and 18·0 % (Table I). The average of the percentage deviations was much less, being 6·8, 8·8 and 7·6 for these organs (Table I). At the same time the maximum percentage differences of the weights of these organs in the pairs of rats are 34·3, 41·8 and 43·5 (Table II).

It, therefore, seems reasonable to infer the positive influence of a testicular extract on each of the mentioned organs if, in a litter of at least four rats, in the two injected rats these organs have increased on an average by at least 40 %, as compared with those of the two uninjected rats, left as controls.

4. *Fluctuations in weight of organs of the castrated rats injected with testicular hormone preparations.*

It is important, in order to estimate the value of this method of assay, to ascertain whether the variations in weight of the injected castrated rats are similar to those of their uninjected litter-mates. For this purpose the percentage deviations from the average weight of each organ in each pair of injected rats is given (Table III), *i.e.* the table is constructed in the same way as Table I. Table IV is constructed in the same way as Table II, *i.e.* it contains the average figures of the percentage difference in weight between the organs of two rats in each pair. (These are the same pairs as used for Table III.)

Altogether 31 pairs of rats were used. The two rats of each pair belonged to the same litter and were injected with the same amount of testicular hormone

Table III. *Percentage deviations of weight of organs from their average weight in each pair of castrated injected rats of the same litter.*

Prostate with seminal vesicles	0·4, 0·7, 0·8, 1·3, 1·5, 1·9, 2·1, 2·5, 2·6, 2·6, 3·6, 3·8, 4·5, 4·7, 4·8, 5·3, 5·7, 5·9, 7·0, 7·4, 7·8, 7·9, 8·0, 11·6, 12·1, 13·7, 15·5, 16·1, 20·0, 29·2, 38·3
	Average = **8·04**
Penis	0, 0, 0·6, 0·8, 0·8, 1·1, 1·5, 1·7, 1·9, 2·6, 2·8, 2·9, 3·4, 3·5, 4·5, 4·7, 5·1, 5·2, 5·6, 7·5, 8·2, 8·3, 8·4, 9·1, 9·3, 10·1, 12·6, 12·9, 14·8, 19·8, 32·1
	Average = **6·5**
Adrenals	0·3, 0·7, 0·8, 0·9, 1·4, 1·6, 1·9, 1·9, 1·9, 2·5, 2·8, 2·8, 2·8, 3·0, 3·1, 3·8, 3·8, 4·2, 4·2, 4·4, 4·7, 5·2, 5·8, 5·9, 7·6, 7·7, 8·7, 9·1, 10·0, 11·7, 14·5
	Average = **4·5**
Thyroid	0·2, 0·5, 0·7, 1·8, 1·9, 2·0, 2·6, 2·8, 3·4, 3·7, 3·8, 4·1, 4·5, 4·6, 5·4, 5·9, 6·6, 6·6, 6·7, 6·8, 7·2, 9·9, 11·0, 11·9, 12·4, 12·5, 13·0, 14·0, 14·7, 15·7, 15·7
	Average = **6·9**
Hypophysis	0·4, 0·4, 0·5, 0·5, 2·3, 2·8, 2·9, 3·1, 3·6, 3·6, 3·9, 4·6, 4·7, 4·8, 5·0, 6·0, 6·0, 6·1, 6·2, 7·2, 8·0, 8·3, 8·9, 10·6, 11·4, 12·2, 13·6, 18·7
	Average = **5·6**
Retroperitoneal fat	0·5, 0·8, 1·0, 2·0, 2·4, 3·9, 5·4, 5·5, 6·6, 7·0, 7·8, 8·2, 8·3, 8·6, 9·1, 10·2, 10·2, 10·3, 12·4, 13·8, 15·0, 15·2, 18·6, 19·0, 19·6, 23·3, 30·1, 34·6, 35·8, 40·6, 56·4
	Average = **14·3**

preparation. Different pairs, however, were injected with different preparations and in varying doses. Only those pairs were taken for the Table which showed a minimum increase of 40 % in the weight of prostate with seminal vesicles as compared with that of the controls of the same litter. The period of injection was from 6 to 14 days. A full account of these experiments will be given elsewhere. In this paper are discussed only the variations in the weights of the organs of the injected rats of the pair, for which purpose further details of the experiments are unnecessary. As can be seen from Table III, the averages of the percentage deviations of weights of organs of injected rats are sufficiently close to those of uninjected rats (Table I). The percentage differences of the weights of organs in the rats of pairs of injected rats are also similar to those of uninjected rats (compare Tables II and IV) with the exception of the high maximum percentage difference in the weight of prostate with seminal vesicles, penis and fat which, however, occurs in a few cases in injected pairs. This can be explained by the fact that the influence of testicular extract is exceptionally strong in a few rats because of their sensitivity to hormonic influence. In addition injections were often made with crude material, insufficiently purified from depressing substances which depress the appetite and therefore the nutrition of the more susceptible animals. Decrease of appetite may occur for example in only one rat of the pair as the result of the injection, or the decrease may be greater in one than in the other with consequent check of the return towards normal of the changes produced. However, as the figures show, this happens in only a few cases (Tables III and IV).

Table IV. *Maximum, minimum and average percentage differences in weight of organs in each pair of injected castrated rats.*

Organs	Average difference	Maximum difference	Minimum difference
Prostate and seminal vesicles	20·8	124·0	0·9
Penis	15·6	95·9	0·0
Adrenals	9·8	34·4	0·7
Thyroid	15·5	38·2	0·4
Hypophysis	13·7	48·1	0·8
Retroperitoneal fat	41·9	259·1	1·1

5. *The test.*

The data from all four tables show, therefore, that for the simple assay of testicular hormones two rats as controls and two rats for injection are sufficient to obtain accurate information with regard to the positive influence of the extract.

For the usual tests, especially when only a small amount of extract is available, injections should be continued for 6 or 7 days. I propose to use the increase in weight of prostate with seminal vesicles as the test of potency. As a rule the weight of the penis is also simultaneously increased, thus corroborating the results obtained on prostate and seminal vesicles, but the penis

responds more slowly to the injections than does the prostate and seminal vesicles, as we shall show in a later paper.

An average increase of 40 % in the weight of the prostate with seminal vesicles of the injected pair, as compared with the control pair, was suggested above as a quite definite proof of the activity of testicular extracts and perhaps as a basic figure for one rat unit. The figure 40 % is a preliminary one, since experiments, which are now in progress, may decrease it.

The figures for percentage deviation from their average weight (Table I) and percentage difference in weights of organs in each pair (Table II) are very close in the case of penis and thyroid to those for prostate and seminal vesicles. Therefore by the same argument one would suggest that an increase by 40 % in the weight of these organs in the injected pair will also clearly prove the activity of the extract on these organs. In the case of the hypophysis and the adrenals the maximum percentage differences in weight of the organs in a pair (Table II) are larger. Perhaps it would, therefore, be advisable for these glands to take a higher figure than 40 % decrease in weight as a definite proof of the activity of testicular extracts, and for the same reason a still higher figure would have to be taken for assays from changes in the weight of retroperitoneal fat under the influence of injections. In this paper a value for these figures is not suggested as has been done for prostate, penis and thyroids, since preliminary work on the influence of injections for a very much longer period than was usual in our experiments would be necessary.

Table V. *Influence of the testicular extracts A and B prepared by two different methods from the same batch of bulls' testes.*

2·5 mg. of the extract were injected twice a day subcutaneously for 7 days. Weights of organs per 200 g. of rat's weight.

Nos. of rats	Injections	Final wt. of rats, g.	Retroperitoneal fat g.	Prostate +sem. ves. mg.	Penis mg.	Adrenals mg.	Thyroids mg.	Hypophysis mg.
1769	Controls	161	3·03	99	111	740	187	129
1773	(uninjected)	159	1·46	91	92	734	189	134
	Average...	**160**	**2·25**	**95**	**102**	**737**	**188**	**132**
1770	Extract A	165	2·35	138	128	573	181	103
1771		164	2·10	134	131	577	183	110
	Average...	**165**	**2·23**	**136**	**130**	**575**	**182**	107
1772	Extract B	160	2·61	140	136	588	228	93
1774		149	1·78	142	164	622	165	117
	Average...	**155**	**2·20**	**141**	**150**	**605**	**197**	**105**

The importance of using rats of the same litter, kept on the same diet, for the different tests, has already been emphasised in previous papers [see, especially, Korenchevsky, 1930].

For an absolutely accurate assay a standard preparation of testicular hormone would have to be used, with which the preparation to be tested would have to be compared. In this case it would be necessary to use a litter of six

castrated rats, two as controls, uninjected, two injected with the standard preparation and two with the new hormone preparation to be tested.

Since a standard preparation is not at present available the following procedure is suggested as the most satisfactory method for ascertaining the strength of the preparation to be tested.

For comparison of the results it is necessary to use rats in litters of the same age and at the same date after castration, belonging to the same stock, reared on the same diet and kept during the experiment on a special ration of the complete synthetic diet mentioned above.

It is preferable to use litters of six castrated rats since they can then be divided into three pairs, one for the controls, uninjected, the other two for the injections of two different doses, or for the comparison of a new preparation with that previously prepared (a substitute for a standard preparation).

As the test of the activity and strength of the preparation a 40 % increase in weight of the prostate with seminal vesicles should be obtained, for which result 6 days of injections should give accurate data, as will be shown in a later paper. For the comparison the weights of the organs calculated per the same unit of rat weight must be used, not the actual figures, which may differ much more according to the differing weights of the rats. An example of the test of two preparations is given in Table V, showing them to be of about the same strength.

Summary.

1. Twenty-five litter-mate pairs of uninjected castrated rats, and thirty-one litter-mate pairs of castrated rats injected with testicular hormone preparations were used.

2. All the rats used for this investigation belonged to the same stock, were reared on the same diet and kept during the experiment on the same paste; but the pairs differed in age and in the period of time since castration.

3. A special method of preparing the organs for accurate weighing was described, by which it was possible to use them afterwards for histological investigation, if necessary.

4. The percentage deviation of weight of prostate with seminal vesicles, adrenals, penis, thyroids, hypophysis and of retroperitoneal fat, from their average weight in each pair, and the percentage differences in weight of these organs in each pair were ascertained.

5. On the average these deviations and differences are not very great and, therefore, the changes in weight of special organs of the pairs of injected rats, as compared with the weight of the same organs of control uninjected rats are suggested for studying the activity and strength of testicular preparations.

6. Since after castration there is a decrease in weight of prostate, seminal vesicles, penis and thyroids and an increase in weight of hypophysis, adrenals and body fat, it is necessary in order to demonstrate the complete activity of

the testicular hormone, or hormones, to obtain a return towards the normal of these organs.

7. However, as a quick, simple and sensitive test, we suggest an increase in the weight of the prostate with seminal vesicles of the injected castrated rats, by at least 40 %, as compared with that of uninjected control rats.

8. For comparison the weights of organs should be used only as calculated per the same unit of rat weight.

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L. THE INFLUENCE OF THE FRESHNESS OF THE TESTES AND OF DESICCATION OF THE TESTICULAR TISSUE ON THE YIELD OF TESTICULAR HORMONE.

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The yield of testicular hormone varies for different batches of bulls' testes [Koch, 1930]. It is, therefore, important to make a study of the conditions which might influence the yield.

It has been shown that strong oxidation destroys the testicular hormone [Dodds *et al.*, 1930; Gallagher and Koch, 1930]. However, several stages in the extraction of the hormone from urine and testes were performed in the presence of air with satisfactory results. Moreover, the testicular hormone survives boiling with acids and with alkalis [Dodds *et al.*, 1930; Gallagher and Koch, 1930]. Dodds and his co-workers have also shown that the activity of the hormone is destroyed by pepsin and by trypsin. This fact suggested to us that the autolytic ferments present in the testicular tissue might also destroy the hormone. Oxidation, and more particularly the influence of autolytic ferments, might both be responsible for the lower yield of the hormone from testes kept for more or less prolonged periods before extraction. The testes supplied from the slaughter-house are of different degrees of freshness and it is a matter of technical difficulty to obtain the testes immediately the bulls have been slaughtered.

It was decided to study the effect of desiccation of the tissue, not only on the yield, but also from a second point of view, that of simplification of the method of extraction. The method of preparation of testicular hormone from bulls' testes which has been much used is that recommended by Koch and Gallagher [1929]. By this method the ground testicular tissue is first extracted with four times its weight of 96 % alcohol, which is afterwards removed by distillation. To save time and expense it was decided to study the influence of drying the tissue so as to obtain the material in such a condition as would permit of a direct benzene extraction of the hormone, without previous alcohol extraction and at the same time without decreasing the yield of hormone. Since the results of our experiments on "fresh" and "kept" testes seemed to

confirm our suggestion that the testicular autolytic ferments are harmful to the hormone, it was decided to destroy the testicular ferments by boiling the minced testicular tissue in water before drying it.

1. THE INFLUENCE OF THE FRESHNESS OF THE TESTICULAR TISSUE.

The greatest care was taken to obtain the testes at the slaughter-house immediately the bulls had been killed. The testes were put into containers surrounded with freezing mixture and brought to the Institute in a frozen condition.

The extracts "Fresh" and "Kept" were prepared in the following way.

The testes were minced, well mixed and divided into two equal parts. The one part, "Fresh," was used for immediate extraction, the second, "Kept," being left in a large open beaker at room temperature for about 20 hours. The "Kept" part was then used for the preparation of extract.

The method used for the extraction of the testicular hormone was that proposed by Koch and Gallagher [1929]. In order to save time the method was not followed to the final purification, being carried out only to the stage of extraction with 70 % alcohol from hexane solution, *i.e.* crude extracts only were prepared. At this stage a preparation is obtained which is readily absorbed from the subcutaneous tissue without producing indurations or a general toxic effect.

The extract obtained was emulsified at about p_H 7·6–7·8, in 1 % gum acacia, to which had been added 0·3 % tricresol, distributed in ampoules and sterilised in the steamer 3 times for 30 minutes on 3 consecutive days.

Experiments were performed on 5 litters containing 19 castrated rats (Table I).

Table I. *Influence of the freshness of the testes on the activity of the testicular extract.*

No. of extract injected	No. of litter	No. of rats in litter	Age of rats in days	No. of days after castration	Initial average weight of rats	Average weight of prostate + seminal vesicles in mg. per 200 g. of rat			Average weight of penis in mg. per 200 g. rat weight		
						Control rats	Rats injected with "Fresh" extract	Rats injected with "Kept" extract	Control rats	Rats injected with "Fresh" extract	Rats injected with "Kept" extract
89	1	5	116	74	254	65	405	146	154	218	183
89	2	2	94	27	260	48	154	—	75	124	—
89	3	2	280	129	399	199	—	291	224	—	258
B.D.H.	4	5	85	62	229	46	114	94	115	—	—
B.D.H.	5	5	93	26	208	184	260	206	136	178	151

Extract No. 89, prepared by us, was injected into the rats of litters Nos. 1 to 3, while extracts prepared by the British Drug Houses, designated in Table I by the letters "B.D.H.," were injected into the litters Nos. 4 and 5.

We have to thank the British Drug Houses and Dr F. H. Carr for very kindly supplying us with the extracts prepared for this experiment, and Dr A. T. Fuller and Dr S.W. F. Underhill for the actual preparation of the extracts.

The period of injection of our extracts was 7 days and of "B.D.H." extracts, 11 days. 10 mg. of the extract were injected subcutaneously into each rat twice a day.

The test used for the determination of the activity and strength of the extract injected, details of which are described in a previous paper [Korenchevsky, 1932], was the increase in weight of the prostate with seminal vesicles of injected castrated rats, as compared with the weight of those of uninjected castrated control rats. This was corroborated by the increase in weight of the penis of injected rats, although the response of the penis to the injections is not so sensitive as that of the prostate with seminal vesicles, as will be shown in a later paper. The results of the experiment are shown in Tables I and II.

Table II. *The yield of the testicular extracts.*

Extracts	Yield in mg. from 1000 g. of testes	Extracts	Yield in mg. from 1000 g. of testes
No. 89 "Fresh"	341	109 "K-G"	561
No. 89 "Kept"	313	109 "Dry"	285
B.D.H. "Fresh"	156	112 "K-G"	996
B.D.H. "Kept"	89	112 "Dry"	550
		124 "K-G"	923
		124 "Dry"	980

As can be seen from the Tables, the injection of both of the extracts prepared by us and of those prepared by the British Drug Houses gave the same results. In litter 1 (Table I) under the influence of "Fresh" extract the weight of prostate with seminal vesicles was increased by 523 % as compared with the uninjected control rats; whilst the "Kept" extract increased the weight of these organs by only 125 %. The respective figures for litters 2 and 3 were 221 % ("Fresh") and 46 % ("Kept"); for litter 4, 148 % and 94 %; and for litter 5, 41 % and 12 %.

The difference between the strengths of the "Fresh" and "Kept" extracts, prepared at the Lister Institute, appears to be much greater than the difference between the strengths of the respective extracts prepared by the British Drug Houses (see Table I). This discrepancy however greatly decreases if the yield is taken into consideration (Table II), the yield from 1 kg. of fresh testes being about double that obtained from kept testes in the "B.D.H." extracts. In our extracts the yield from fresh testes was only slightly greater than from kept material.

In litter 5, the "B.D.H." extracts were much less active than in litter 4. This was explained by the fact that the experiments on litter 5 were started 2 weeks later than those on litter 4; consequently the extract had been kept 2 weeks longer in the ampoules. This indicates that the extract loses much of its activity when kept as a weak emulson in 1 % gum acacia in the presence of air, which probably oxidises the hormone. Moreover we have been able to show the presence of oxidising ferments in the 1 % gum acacia solution used for emulsifying the hormone. These facts suggest that 1 % gum acacia is unsuitable if the hormone is to be preserved for long periods in emulsion form.

The results obtained show quite definitely that the yield of active testicular hormone is much greater from fresh testes than from testes kept for about 20 hours at room temperature.

2. The influence of drying the testicular tissue, previously boiled, on the yield of the hormone.

Bulls' testes were minced, well mixed and divided into two equal parts. One part was extracted by the method of Koch and Gallagher in the usual way to the same stage of purification as in the previous experiment, *i.e.* the final extract was prepared by extraction with 70 % alcohol from hexane solution.

The other part of the minced testes was thrown into about twice its volume of boiling water, acidified with 50 % acetic acid to p_H 4·6–4·8. The mixture was boiled for 15 to 20 minutes, by which time the proteins had coagulated and also most of the ferments had been destroyed. The mixture was then filtered through a Büchner funnel and pressed out if necessary. The precipitate was then spread on sheets of glass, placed in the hot room at 37° and dried in a gentle current of air produced by an electric fan. A powerful current of air seems to be injurious to the activity of the preparation. If stirred occasionally, the tissue dries in about 5 to 12 hours, the speed of drying depending chiefly on the degree of removal of water from the boiled tissue by the press and the thinness of the layer on the glass.

A light brown, porous, brittle, dry mass was obtained, which was extracted twice with benzene (24 hours in all). The extraction proceeds very quickly because of the porous nature of the dried tissue, due to the coagulation of the proteins, which not only makes the extraction quicker and easier but also helps to hasten the process of drying.

The benzene extract was then treated by Koch and Gallagher's method to the same stage of purification as was the first portion of testicular tissue.

In what follows we shall designate the extract prepared by the Koch-Gallagher method as "K-G" extract and the extract prepared by the method as modified by us as "Dry" extract.

By the method detailed above we prepared, from three different batches of testes, three different pairs of the extracts "K-G" and "Dry." The strengths of these extracts were tested in the same way as in the previous experiments, and the results are summarised in Tables II and III.

10 mg. of the extracts were injected twice a day into the litters 5, 7 and 8, for 10, 9 and 12 days respectively.

As is shown by Table IV, in the first two batches prepared (extracts Nos. 109 and 112) the yield of "Dry" extract was less than that of "K-G" extract. The weights of prostate with seminal vesicles, and of penis, however, indicate that the strength of the same weight unit of the "Dry" extract was greater than that of the "K-G" extract (Table III) showing that, in these cases, a purer and more concentrated extract had been prepared by the "Dry"

method. From the third batch (extracts No. 124) the yields by the two methods were nearly the same (Table II), but the strengths were also nearly the same (Table III), being slightly greater in "Dry" extract than in "K-G" extract.

Table III. *Influence of desiccation of the testicular tissue on the activity of the testicular hormone.*

No. of extract injected	No. of litter	No. of rats in litter	Age of rats in days	No. of days after castration	Initial average weight of rats	Average weight of prostate +seminal vesicles in mg. per 200 g. of rat			Average weight of penis in mg. per 200 g. rat weight		
						Control rats	Rats injected with "K-G" extract	Rats injected with "Dry" extract	Control rats	Rats injected with "K-G" extract	Rats injected with "Dry" extract
109	6	4	92	54	229	84	124	153	90	109	134
112	7	5	81	55	199	54	118	131	73	107	121
124	8	7	45	10	92	109	207	215	82	127	146

Table IV. *The influence of the testicular extracts 124 "K-G" and 124 "Dry" on the sexual organs of castrated rats of litter No. 8.*

No. of rat	Group of rats	Weight of prostate +seminal vesicles in mg.		Weight of penis in mg.		Final weight of rats g.
		Actual	Per 200 g. of rat	Actual	Per 200 g. of rat	
1820	Controls	66	116	53	93	114
1821		82	101	58	71	163
	Average	74	109	56	82	139
1822	Test. ext. 124 "K-G"	183	208	111	126	176
1823		145	205	90	127	142
	Average	164	207	101	127	159
1824	Test. ext. 124 "Dry"	151	217	104	150	139
1825		170	217	105	134	157
1826		162	212	117	153	153
	Average	161	215	109	146	150

Differences in the completeness of coagulation of the proteins during the boiling process and in the degree of drying of the testicular tissue probably explain the difference in concentration of the testicular hormone in the "Dry" extracts Nos. 109, 112 and 124. The time of drying and the strength of the current of air produced by the electric fan might also have some influence. The yield of the final testicular preparations also varies for different batches (Table II). We rigorously followed the same procedure in every detail in preparing each pair of extracts from a batch of testes. The details however were slightly modified for different batches of testes. For example, in batches 89 and 109, 70 % alcohol by volume was used for the final extraction from hexane solution, whilst in batches 112 and 124, 70 % alcohol by weight was used. The extraction of the preparation from the hexane solution by 70 % alcohol was in some batches made only twice, in others more often. These results will be discussed elsewhere.

From the results obtained it is clear that it is possible to extract from the testicular tissue the same amount of testicular hormone by our modification of the method as by the original method. In order to show how the average figures of Tables I and III were obtained we give in Table IV the detailed figures for the comparison of the strengths of extracts No. 124 "K-G" and No. 124 "Dry."

Conclusions.

1. The freshness of bulls' testes plays an important part in the yield of the hormone.

2. After boiling the testicular tissue in water acidified with acetic acid to p_H 4·6–4·8, it is possible to dry it at 37° without appreciably decreasing the yield of the hormone.

3. Apart from oxidation, the autolytic ferments present in the testicular tissue are probably the most destructive agents of the testicular hormone in testes removed from the organism and kept at room temperature.

4. A simplification of the method of extraction of the hormone is possible by benzene extraction from testicular tissue, which has been first boiled and then dried at 37°.

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LI. THE INFLUENCE OF CRYPTORCHIDISM ON THE GASEOUS AND NITROGENOUS METABOLISM OF RATS.

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The chief difference between the testes of cryptorchid and of normal animals is the atrophy or complete disappearance of the seminiferous cells. In previous papers [Korenchevsky, 1930; Korenchevsky and Dennison, 1930] it was shown that most rats become obese after being made cryptorchid, although to a less degree than after castration. The development of obesity in cryptorchid animals was, therefore, ascribed mainly to atrophy of the seminiferous cells. Taking into consideration other data obtained it was suggested that the seminiferous cells (as well as Leidig's cells) produce testicular hormones and that the hormones of the seminiferous cells stimulate metabolism. An explanation was also offered as to why the degree of obesity developed by different cryptorchid and castrated animals varies, or, in some cases, is even absent [Korenchevsky, 1925, 1930].

Present investigation.

The aim of this investigation is to ascertain the influence of cryptorchidism on the metabolism, especially during the period of resorption of the seminiferous tissue from the testes of rats made cryptorchid. This period starts a few days after the operation and continues for different periods of time in rats of different ages, being completed in from 3 to 7 months. By the end of this period the testicular cells consist of Sertoli cells, in very atrophied tubules, and of well preserved or even hypertrophied Leidig's cells.

Technique.

Successful experiments were performed on 10 rats, in 5 of which both the gaseous and nitrogenous metabolisms were investigated, and in the other 5 the nitrogen metabolism only. The technique used has been described in a previous paper [Korenchevsky and Dennison, 1932].

Before the operation the normal metabolism was investigated during a period of 2 days, and the investigation repeated 2 or 3 times at intervals of about 10 days. The animals were then made cryptorchid, after which the

metabolism was again investigated at different periods after operation, each time for 2 or more days. Only the averages of the results for each of the periods are given in the Tables.

The operation was performed in the same manner as in the previous investigation [Korenchevsky, 1930]. As can be seen from Table I, adult rats only were chosen. Those, however, used for gaseous and nitrogenous metabolism (first five, Table I) were older rats and, during the period of the experiment,

Table I. *Weight of rats (g.) during different periods after cryptorchidism.*

No. of rat	Normal period	No. of days after operation						
		6–17	20–29	33–38	42–59	69–84	122–136	196
986	334	332	333*	335*	336	—	—	—
1241	366	343	342	345*	348	—	—	—
1243	355	352	351	358*	363	366	—	—
1596	371	363	360	355	358	364	372	384
1597	365	361	361*	362	363	364	366	—
596	340	345	350	350	—	—	—	—
597	369	379	384	391	—	—	—	—
679	260	262	266	278	—	—	—	—
681	304	302	315	330	—	—	—	—
682	260	264	272	281	—	—	—	—
Average	332	330	333	339	—	—	—	—

* Interpolated from all available data.

increased in weight not at all or only very slowly. The remaining 5 rats, used only for nitrogenous metabolism, were younger and growing more rapidly. The food ration was smaller for the former rats than for the latter, being on the average 110 g. and 166 g. of paste respectively (per week per 200 g. of rat weight).

Changes in metabolism.

As can be seen from Table II the gaseous metabolism definitely increased, the highest figures being obtained during the first two periods, *i.e.* from 6 to 38 days after the operation of cryptorchidism had been performed. This period coincides with the period of greatest resorption of the seminiferous cells, while they are still in abundance. After this maximum point the gaseous metabolism returned towards normal and, if the experiment were of sufficiently long duration (rat 1596), returned to the normal level. The respiratory quotient showed no great changes.

The nitrogenous metabolism (Table III) decreased slightly on the average during the period of increased gaseous metabolism. The nitrogen balance remained positive throughout, in some cases not only maintaining but even exceeding, the normal level.

Taking into consideration this fact and also that the weight, in spite of the constant food ration, was increasing in most cases, and in others was returning to or had reached normal, it must be admitted that the rats were growing. The nitrogen in this case must have been retained and used in the body chiefly for purposes of growth.

Table II. *Gaseous metabolism during different periods after cryptorchidism.*

CO_2, O_2 and H_2O in g. per week per 200 g. of rat weight.

No. of rat	Normal period	Days after operation 6–17	20–38	42–59	69–84	122–136	196
			Average H_2O excretion.				
986	90·4	109·2	103·6*	96·4	—	—	—
1241	89·7	104·1	111·8	93·5	—	—	—
1243	89·4	91·3	88·1	76·5	102·0	—	—
1596	109·8	103·3	112·2	113·6*	111·4	110·0	98·6
1597	103·9	103·6	101·6	99·2*	95·8	99·6	—
Average	96·6	102·3	103·5	95·8	—	—	—
			Average CO_2 excretion.				
986	52·4	58·4	57·0*	55·2	—	—	—
1241	60·7	65·2	66·3	63·4	—	—	—
1243	53·0	63·2	64·1	60·6	59·5	—	—
1596	61·5	63·7	64·6	64·3*	63·1	62·1	61·8
1597	59·6	64·0	63·7	63·6*	63·2	62·3	—
Average	59·4	62·9	63·1	61·4	—	—	—
			Average O_2 consumption.				
986	44·1	49·9	48·4*	46·8	—	—	—
1241	50·3	55·2	55·6	53·9	—	—	—
1243	49·0	53·0	54·2	51·4	49·8*	—	—
1596	50·9	51·9	54·4	53·9*	54·3	52·0	51·0
1597	48·9	53·5	54·5	53·8*	52·7	51·7	—
Average	48·6	52·7	53·4	52·0	—	—	—
			Respiratory quotient.				
986	0·87	0·85	0·85*	0·86	—	—	—
1241	0·88	0·86	0·87	0·86	—	—	—
1243	0·86	0·87	0·86	0·86	0·87	—	—
1596	0·88	0·89	0·87	0·88*	0·85	0·87	0·89
1597	0·87	0·87	0·85	0·86*	0·88	0·88	—
Average	0·87	0·87	0·86	0·86	—	—	—

* Interpolated from all available data.

Discussion.

The maximum increase of gaseous metabolism occurred during the period of greatest resorption of the seminiferous cells, while they were still abundant in the cryptorchid testes. This indicates that during the process of resorption substances were liberated from the seminiferous cells which stimulated the gaseous metabolism. It seems reasonable to suggest that these substances were hormones produced by the seminiferous cells. This conclusion is in agreement with the fact which was mentioned above, that most cryptorchid animals finally become obese if given food *ad libitum* [Korenchevsky, 1930].

There is one important disadvantage in experiments of very long duration on animals which are still growing, if the amount of food has to remain constant as is the case in metabolism experiments. With time the chemical composition of such animals changes, since the large stores of fat, carbohydrates, proteins

Table III. *Nitrogenous metabolism during different periods after cryptorchidism.*

N in g. per 200 g. rat weight per week.

No. of rat	Normal period	No. of days after operation 6–17	20–29	33–38	42–59	69–84	122–136	159–196
			Average N excretion in urine.					
986	1·05	0·98	1·00*	1·02*	1·05	—	—	—
1241	1·10	1·16	1·09	1·19*	1·26	—	—	—
1243	1·11	1·14	1·08	1·09*	1·09	1·06	—	—
1596	1·39	1·26	1·56	1·64	—	1·28	1·25	1·41
1597	1·79	1·60	1·61*	1·62	—	1·69	1·69	1·59
596	1·74	1·70	1·52	1·55	—	—	—	—
597	1·59	1·58	1·57	1·51	—	—	—	—
679	1·88	1·68	1·70	1·50	—	—	—	—
681	1·44	1·41	1·35	1·33	—	—	—	—
682	1·71	1·62	1·71	1·69	—	—	—	—
Average	1·48	1·41	1·42	1·41	—	—	—	—
			Average N balance.					
986	+0·09	+0·16	+0·13*	+0·09*	+0·06	—	—	—
1241	+0·13	+0·14	+0·22	+0·09*	+0·01	—	—	—
1243	+0·19	+0·16	+0·22	+0·20*	+0·16	+0·19	—	—
1596	+0·25	+0·40	+0·11	+0·06	—	+0·42	+0·38	+0·13
1597	−0·09	+0·10	+0·09*	+0·08	—	+0·04	±0·00	+0·07
596	±0·00	−0·02	+0·14	+0·11	—	—	—	—
597	+0·16	+0·08	+0·08	+0·10	—	—	—	—
679	+0·10	+0·22	+0·18	+0·31	—	—	—	—
681	+0·25	+0·24	+0·23	+0·18	—	—	—	—
682	+0·17	+0·28	+0·14	+0·09	—	—	—	—
Average	+0·13	+0·18	+0·15	+0·13	—	—	—	—

* Interpolated from all available data.

and other constituents, deposited in the body during abundant feeding, are decreased or may even in some cases be exhausted. These are used for the most part for the purpose of building the growing tissues. The result is that the unit of weight of the animal at the beginning and end of the experiment is of a different nature, consisting at the beginning, to a considerable extent, of stores of fat, carbohydrate and protein, which are inert in the metabolism. By the end of the experiment these passive stores are converted into living cells which are very active in metabolic exchange, so that nearly the whole weight of the animal becomes active in its metabolism. In order that the animal may continue to live in a normal state, *i.e.* at the normal metabolic rate, for the same unit of its weight, only one way is available, namely, to reduce, if possible, its metabolism. Otherwise the weight will decrease, because the constant food supply will become insufficient for the increased amount of living tissues. Taking all these facts into consideration it must be admitted that the rats in these experiments, after being made cryptorchid, did in fact, by the end of the experiment, decrease their metabolism.

After the period of increased gaseous metabolism, due to abnormally great absorption of the hormones of the seminiferous cells, the weight of the animals returned towards or reached normal, and even in some cases increased; the

gaseous metabolism also returned towards or actually reached the normal value.

Moreover the nitrogen balance remained positive and in some cases even showed an increased nitrogen deposition, thus allowing growth of the tissues. All these metabolic changes occurred in spite of the constant quantity of food sufficient to maintain both the weight and the metabolism of the animal at a constant level, but not exceeding its needs. Thus the metabolism results corroborate the results obtained from the chemical investigation of rats [Korenchevsky and Dennison, 1930], when an increased amount of solids was found in the tissues of cryptorchid animals. This increase was explained by an increase in the deposition of fat and of nitrogenous substances.

The only probable explanation of these metabolic changes seems to be the decrease in metabolism due to the absence of the metabolism-stimulating hormones of the seminiferous cells.

If the ration of food is only sufficient for the equilibrium of the animal, there is a limit to the time during which even the decreased metabolism will satisfy the needs of the increasing amount of living tissue of the growing animal. We were, therefore, unable to keep the growing cryptorchid animals on the same amount of the same food for longer than about 10 months.

In order to obtain accurate results from such experiments as are mentioned above, care must be taken to find the right food ration, in quantity and quality, sufficient to maintain both the weight and metabolism of the animal at a constant level, but not to exceed its needs.

Summary.

1. The gaseous and nitrogenous metabolisms before and after the operation of cryptorchidism were studied in 5 rats, and the nitrogenous metabolism only in another 5 rats. The period of observation lasted from 38 to 200 days after the operation.

2. An increase in the gaseous metabolism was observed during the period of most active and abundant resorption of the seminiferous cells from the cryptorchid testes.

3. This corrobrates the suggestion made elsewhere as the result of other experiments, that the seminiferous cells secrete a testicular hormone which stimulates metabolism.

4. In all the rats, during the whole period of observation, in spite of the constant daily ration of food, deposition of nitrogen in the body was observed, in some rats remaining at or exceeding the normal level, even during the period of increased gaseous metabolism.

5. These data confirm the previous results of the chemical investigation of cryptorchid animals, in the tissues of which an increased amount of fat and of nitrogenous substances was noted.

6. Data are discussed which indicate a decreased metabolism in cryptorchid rats after the completion of the period of absorption of the seminiferous cells.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due.

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LII. THE METABOLISM OF *i*-CYSTINE IN THE RAT.

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(*Received March 1st, 1932.*)

The present work was undertaken in view of the fact that very little information is available regarding the metabolism of *i*-cystine, and because it was felt that a knowledge of the differences in behaviour between *l*- and *i*-cystine in the animal organism might throw light both upon the structure of *i*-cystine and upon the metabolism of this amino-acid.

Neuberg and Mayer [1905] observed the utilisation of *i*-cystine by *Aspergillus niger* and by *Penicillium*. Gortner and Hoffman [1927] observed its utilisation by *A. niger*. Hele and Pirie [1931] have studied the oxidation of *i*-cystine in the dog, and their findings are very similar to those described in this paper for the case of the rat. In their papers on the resolution of *i*-cystine into its *l*- and *d*-isomerides, du Vigneaud and Hollander [1930, 1931] state that they are undertaking the study of the metabolism of *d*-cystine, and their results are awaited with keen interest.

In order to determine to what extent *i*-cystine can replace the naturally occurring amino-acid for purposes of tissue formation, the technique elaborated by Sherman and Woods [1925] was adopted.

I. The oxidation of *l*- and *i*-cystine in the rat.

Experimental.

The *l*-cystine used in these experiments was prepared by acid hydrolysis of human hair, and the *i*-cystine by boiling this *l*-cystine with hydrochloric acid, according to the method of Hoffman and Gortner [1922].

Eight adult male rats were taken from the inbred piebald strain used in this laboratory. Their combined weight at the beginning of the experiment was 1900 g., and at the end 2010 g. A daily ration of 15 g. of synthetic diet per rat was fed throughout the experiment. The composition of this diet and the methods employed for collection and analysis of urine were identical with those described in a previous communication [Lawrie, 1931].

On the days when a dose of cystine was to be given, the cystine was thoroughly mixed with the day's ration of synthetic diet, and the mixture was always readily eaten. The influence of this dosage upon the sulphur partition

of the urine is shown in Fig. 1, and the extent to which the ingested cystine had been oxidised to inorganic sulphate is given in Table I.

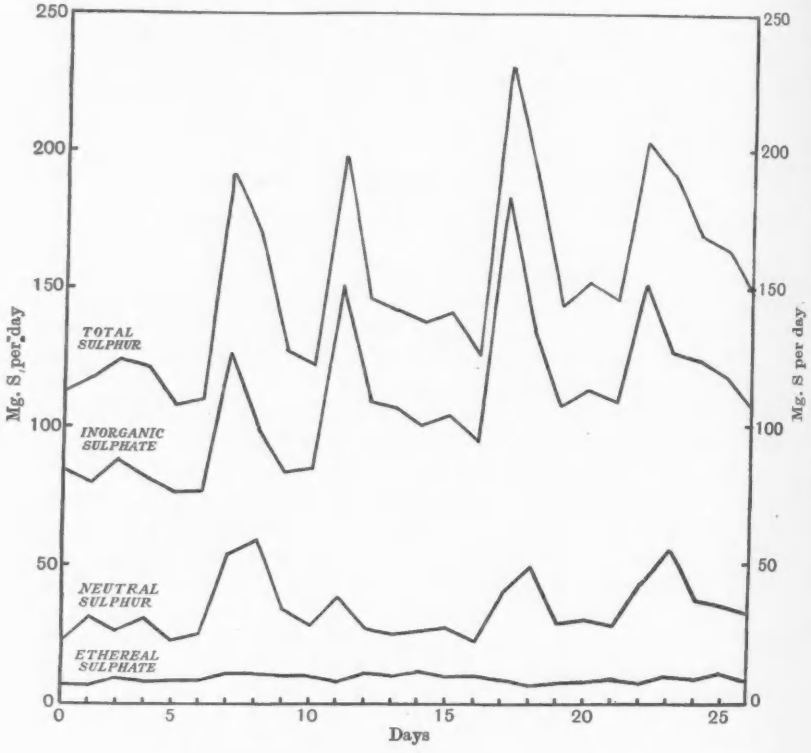

Fig. 1.

Dosage. On 7th day, 0·7 g. *l*-cystine mixed with diet.
On 11th day, 0·7 g. *i*-cystine " "
On 17th day, 1·0 g. *i*-cystine " "
On 22nd day, 1·0 g. *l*-cystine " "

Table I.

	Extra inorganic sulphate excreted in 24 hours after dose		Extra inorganic sulphate excreted in 48 hours after dose		Extra inorganic sulphate excreted in 96 hours after dose	
Dose	mg. S	% dose	mg. S	% dose	mg. S	% dose
700 mg. of *l*-cystine	45	49	63	68	—	—
1000 mg. of *l*-cystine	41	31	57	43	78	59
700 mg. of *i*-cystine	67	72	—	—	—	—
1000 mg. of *i*-cystine	79	59	112	84	—	—

Discussion of results.

Reference to Fig. 1 will show that during the 24 hours following each dosage with cystine there is a marked rise in the rate of excretion of inorganic sulphate. A fall in the rate of excretion of this fraction then follows, which, although rapid during the second, and sometimes during the third, period of 24 hours after the dosage, subsequently becomes much slower. The rate of inorganic sulphate excretion then becomes comparatively steady at a higher level than before the dose was given. Since no similar prolongation of the effect due to dosage is seen in the neutral sulphur and ethereal sulphate fractions, and since the animals were slightly gaining in weight throughout the experiment, whilst being restricted to a constant food intake, it is very probable that the tendency of the inorganic sulphate fraction to increase throughout the experiment is due to retention, and slow subsequent excretion, of a part of the sulphur given as cystine.

In order, therefore, to obtain as clear an indication as possible of the rate and extent of the oxidation of any dose, it is necessary to compare the rise in inorganic sulphate excretion immediately following the giving of that dose, with the level of this fraction during those days immediately preceding the dosage which showed a steady rate of excretion of inorganic sulphate. Hence the extra inorganic sulphate excretion following the first dosage is calculated from the average values of the inorganic sulphate fraction for the first 6 days of the experiment. The extra inorganic sulphate produced by the second dose is estimated by comparison with the average of the inorganic sulphate values for the ninth and tenth days of the experiment. Similarly, the values averaged for the estimation of the effect of the third dose were those of the twelfth to the sixteenth days of the experiment; and the effect of the last dose was estimated from the average of the values of the inorganic sulphate fractions on the nineteenth, twentieth and twenty-first days of the experiment. From the above considerations it is clear that the figures for the 24 hours following dosage are likely to give the most reliable indication of the readiness with which the rat oxidises the cystine isomeride in question.

The figures so obtained are given in Table I, and from them it is clear that *i*-cystine is oxidised by the rat at least as readily as *l*-cystine. In fact, the figures suggest a more complete oxidation of the *i*- than of the *l*-isomeride, and this relationship might be explained by the greater solubility of the former [Hoffman and Gortner, 1922].

A transient rise in the rate of excretion of neutral sulphur followed the administration of each dose of cystine. This rise was less marked after dosage with *i*-cystine than after *l*-cystine. This observation again indicates that the former isomeride has been more readily metabolised.

II. A comparison of the values of *l*- and *i*-cystine as supplements for a diet deficient in cystine.

As a basal diet in which cystine deficiency is the factor limiting growth rate, the diet described by Sherman and Woods [1925] was used with the following slight modifications. The salt mixture was replaced by that used in this laboratory, and in some of the later experiments the percentage of cod-liver oil was raised to 1·65. The dried milk powder was supplied by Messrs Glaxo, Ltd.

With the exception of 6 albino rats supplied by the above firm, and referred to in this paper as "litter 2," all the animals used in this experiment were bred in this laboratory, and were of the same strain as those used in Part I of this work.

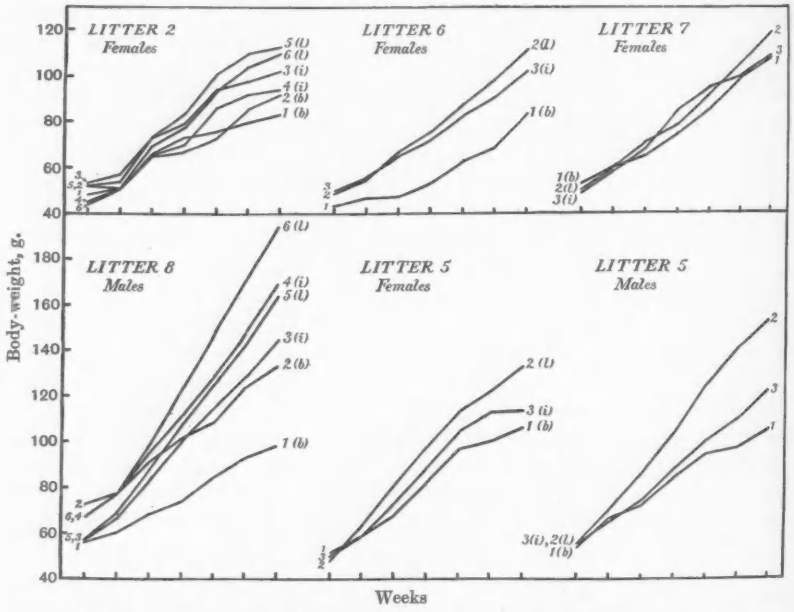

Fig. 2. Growth curves of rats fed upon a basal diet deficient in cystine, marked (*b*); and upon the same diet supplemented with 5 g. of *i*-cystine per 10,000 g. of dry diet, marked (*i*); and supplemented with the same proportion of *l*-cystine, marked (*l*).

In a preliminary experiment, the growth rates of rats fed upon the basal diet were compared with those of rats receiving additions to their diet of 1, 2·5, 5, and 10 g. per 10,000 g. of dry diet, of *l*- and *i*-cystine respectively. The results obtained confirmed the findings of Sherman and Woods that addition of *l*-cystine to the basal diet caused an increase in growth rate over a period of 6 weeks which is proportional to the percentage of cystine added, until 5 g. of cystine per 10,000 g. of dry diet have been added. They also indicated that

in order to obtain a rate of growth equal to that shown by rats fed upon the basal diet *plus* *l*-cystine in proportions up to 5 g. per 10,000 g. of dry diet, approximately twice the percentage of *i*-cystine must be added to the diets of control rats receiving the basal diet.

The diet was therefore suitable for a comparison between *l*- and *i*-cystine; and in the remaining experiments litter-mates, of the same sex, and started upon experiment at the same time, were divided into the following three groups. Group 1 received the basal diet. Group 2 received the same diet *plus* 5 g. of *l*-cystine per 10,000 g. of dry diet, whilst group 3 received the same basal diet supplemented with 5 g. per 10,000 g. dry diet of *i*-cystine. These diets were all fed *ad lib.* for a period of 6 weeks.

The differences in growth rate produced by feeding these three diets to litter mates are shown graphically in Fig. 2. Table II shows the growth rates calculated in grams per week; the increase in growth rate shown by each animal over its litter-mate fed upon the basal diet; and the ratio (expressed as a percentage) of this increase, for each animal of group 3 to the corresponding increase shown by its litter-mate of the same sex in group 2.

Table II. *Growth rates of rats over a period of 6 weeks, expressed as g. per week.*

	Diet					
Litter no. and sex	Cystine-low basal diet Column A	Cystine-low basal diet + 5/10,000 of *i*-cystine Column B	Cystine-low basal diet + 5/10,000 of *l*-cystine Column C	B − A	C − A	$\frac{B-A}{C-A} \times 100$
1♀	9·0	—	—	—	—	—
2♀	(1) 5·8 } Av. (2) 6·7 } 6·3	(3) 8·2 } Av. (4) 8·2 } 8·2	(5) 10·2 } Av. (6) 11·0 } 10·6	1·9	4·3	44
3♀	5·9	—	—	—	—	—
4♂	—	(3) 13·3 } Av. (2) 16·7 } 15·0	(1) 18·5	—	—	—
5♀	(1) 9·2	(3) 10·8	(2) 14·4	1·6	5·2	31
5♂	(1) 8·7	(3) 11·2	(2) 16·3	2·5	7·6	33
6♀	(1) 6·8	(3) 8·8	(2) 10·8	2·0	4·0	50
7♀	(1) 9·0	(3) 10·2	(2) 11·5	1·2	2·5	48
8♂	(1) 7·0 } Av. (2) 10·2 } 8·6	(3) 14·5 } Av. (4) 16·7 } 15·6	(5) 17·7 } Av. (6) 21·2 } 19·5	7·0	10·9	64
						Av. 45

Note. The figures in brackets serve to distinguish litter-mates of the same sex in Fig. 2.

Discussion of results.

The results given in Fig. 2 and Table II show clearly that *i*-cystine is able to supplement a diet deficient in cystine. They also show equally definitely that it is not so efficient for this purpose as the normally occurring *l*-cystine. Under these circumstances one may conclude that the rat is capable of resolving *i*-cystine, and using the *l*-isomeride, for tissue building, whilst the *d*-cystine is excreted, probably after complete oxidation of its sulphur. That the difficulty

experienced by the rat in utilising *i*-cystine for purposes of tissue building arises subsequent to its absorption from the alimentary canal is rendered very probable by the results given in Part I of this paper.

Previous workers have described a persistence of a type of fur characteristic of young rats and the occurrence of symmetrical areas of baldness in rats fed upon diets deficient in cystine. These effects have been noticed in the present experiment in some of the rats of groups 1 and 3.

Cystine and tryptophan appear to be markedly different in respect to the relationships which they show between their optical activity and nutritional values; for Berg and Potgieter [1932] have very recently shown that the feeding of *dl*-tryptophan is as efficient in promoting growth in rats fed upon a diet deficient in tryptophan, as is the feeding of the same quantity of the naturally occurring *l*-tryptophan.

SUMMARY.

The rat can oxidise *i*-cystine as rapidly and completely as *l*-cystine.

The value of *i*-cystine for tissue building purposes in the rat lies between one-third and two-thirds of that of *l*-cystine.

I wish to express my gratitude to Sir F. G. Hopkins for his interest in this work, and to Dr T. S. Hele and Dr L. J. Harris for advice in connection with the experimental technique.

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LIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXIII. PUBERULIC ACID $C_8H_6O_6$ AND AN ACID $C_8H_4O_6$, NEW PRODUCTS OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM PUBERULUM* BAINIER AND *PENICILLIUM AURANTIO-VIRENS* BIOURGE. WITH AN APPENDIX ON CERTAIN DIHYDROXY-BENZENEDICARBOXYLIC ACIDS.

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In a biochemical and toxicological study of *Penicillium puberulum* Bainier, one of the organisms responsible for the deterioration of maize, Alsberg and Black [1913] isolated from the products of metabolism of this mould when grown on Raulin's medium a definite crystalline substance, to which they gave the name of penicillic acid. They determined its empirical formula ($C_8H_{10}O_4$) and described its properties in detail, but did not determine its constitution. Since the authentic culture used by Alsberg and Black was available to us through the courtesy of Dr Charles Thom, of the United States Bureau of Agriculture, it was decided to investigate further this interesting organism, the morphological characteristics of which are described on p. 272 of Thom's Penicillia [1930].

It soon became evident that *P. puberulum* produces, in addition to penicillic acid, small quantities of a variety of other products, some of which are new, and it is our purpose here to describe and characterise one of these products, an acid having the empirical formula $C_8H_6O_6$, and yielding a deep reddish-brown colour with ferric chloride. We propose to give to this acid the name "puberulic acid."

30 litres of Czapek-Dox medium of the following composition were made up. Glucose 50·0 g.; $NaNO_3$ 2·0 g.; KH_2PO_4 1·0 g.; KCl 0·5 g.; $MgSO_4, 7H_2O$ 0·5 g.; $FeSO_4, 7H_2O$ 0·01 g.; distilled water 1000 cc.

5 litres of this medium were distributed in each of 6 silica trays contained in the steriliser-incubator described by Birkinshaw, Charles, Lilly and Raistrick [1931]. After sterilisation, the medium was sown with a spore suspension of *P. puberulum* and incubated at 24°.

Sterile air was passed continuously over the surface of the mycelium at the rate of 4·5–6 cu. ft. per hr. After 12 days' incubation, the metabolism solution, which gave a brownish-red colour with ferric chloride, was removed and filtered.

The total solution, containing 0·58 % of residual glucose, and having a volume of 23·65 litres, was evaporated *in vacuo* and filtered from a yellowish-brown substance, which separated on evaporation. This substance proved to be the magnesium salt of an acid having the empirical formula $C_8H_4O_6$, which will form the subject of a future communication. The filtrate was acidified with a known amount of sulphuric acid and extracted with ether. The ether extract deposited on cooling a certain amount of yellow material which was separated, and weighed 1·5 g. This product, which forms the subject of the present communication, will be called puberulic acid.

The ethereal mother-liquor was evaporated, the residue taken up in water, filtered from a small amount of insoluble matter, and again extracted with ether, from which crystals finally separated. This product was found to agree with that described by Alsberg and Black under the name of penicillic acid.

During the progress of this work it was observed that a brownish-red colour reaction was also obtained by the addition of ferric chloride to the metabolism solution from another mould species, *P. aurantio-virens* Biourge, when grown on Czapek-Dox glucose solution. This culture was kindly supplied by Prof. Philip Biourge of the University of Louvain. It is Biourge's culture number B. 77, and its morphological characteristics are described by him [1923, p. 119] and by Thom [1930, p. 316]. Since this organism appears to give larger yields of both puberulic acid and the acid $C_8H_4O_6$ already referred to than does *P. puberulum*, it was used for the preparation of these two substances.

As the result of a large number of experiments with Czapek-Dox media, containing varying amounts of glucose and mineral salts, the following medium, for which we are indebted to our colleague Mr George Smith, was finally chosen as giving the best yields of puberulic acid and the acid $C_8H_4O_6$.

Glucose 70·0 g.; $NaNO_3$ 15·0 g.; KH_2PO_4 1·0 g.; KCl 0·5 g.; $MgSO_4, 7H_2O$ 0·5 g.; $FeSO_4, 7H_2O$ 0·005 g.; distilled water 1000 cc.

This was distributed in 350 cc. quantities in 1 litre conical flasks, sterilised, sown with a spore suspension of *P. aurantio-virens* and incubated at 24° for 42 days.

It was necessary, however, to modify the method employed for the isolation of the acids owing to the presence in the metabolism solution of a large amount of succinic acid, which was extracted by ether from the evaporated solution along with the other acids. The method adopted depends upon the observation that both puberulic acid and the acid $C_8H_4O_6$ are precipitated as deep red insoluble nickel salts on the addition of nickel sulphate solution to the neutralised metabolism solution, whereas succinic acid remains in solution.

The filtered metabolism solution was therefore first neutralised to phenolphthalein by the addition of 2*N* NaOH, the amount required being determined

by titration. Puberulic acid and the acid $C_8H_4O_6$ were then precipitated by the addition of a slight excess of *M* nickel sulphate solution, the amount added being usually about equal in volume to the NaOH added. After the bright red precipitate had settled, most of the liquid was siphoned off, and the precipitate filtered and washed. It was then ground up with an excess of 2*N* hydrochloric acid. The colour now changed from a bright red to a dirty yellow—the latter representing the colour of the crude acids. The mixture was then extracted with ether, and the evaporated ether extract taken up in a little methyl alcohol and allowed to crystallise. By this means two or three crops of a bright yellow colour were obtained of a mixture of puberulic acid and the acid $C_8H_4O_6$.

These two acids are difficult to separate by fractional crystallisation, but may be satisfactorily separated by taking advantage of the fact that, on treating the mixture of crude acids with acetic anhydride and sodium acetate, the acid $C_8H_4O_6$, which does not form an acetyl compound, separates from the diluted acetylation mixture as the acid sodium salt, whereas puberulic acid forms a diacetyl compound which remains in solution and may be precipitated by acidification with a mineral acid.

In a typical experiment the total crude mixed acids from 143 flasks (50 litres of medium), weighing 16·4 g., were heated with 33 g. of anhydrous sodium acetate and 65 cc. of acetic anhydride for 20 minutes, in an oil-bath, the temperature of which was maintained at 140–150°. The still warm mixture was poured into about 300 cc. of water and stirred. The whole dissolved, giving a deep yellow-brown solution, from which the acid sodium salt of the acid $C_8H_4O_6$ rapidly began to crystallise. After standing overnight, this was filtered off and dried, and weighed 8·5 g. The filtrate was acidified with 42 cc. of 10*N* sulphuric acid. The crude diacetylpuberulic acid separated as a somewhat dark crystalline solid which weighed 7·0 g. Most of the colour disappeared on washing with a little hot absolute alcohol. The product was recrystallised twice from the same solvent with the addition of a little animal charcoal. The final product, consisting of colourless needles, weighed 3·6 g. and melted at 212°.

This product, when mixed with a sample of diacetylpuberulic acid obtained from *P. puberulum*, showed no depression in melting-point, thus indicating the identity of the two products.

Puberulic acid, $C_8H_6O_6$, was obtained by gentle hydrolysis of the pure diacetyl compound (2·97 g.) by boiling with 150 cc. of *N* NaOH in an atmosphere of nitrogen for 20 minutes. The hydrolysis mixture was cooled and acidified with 15·5 cc. of 10*N* H_2SO_4, when the free acid separated as a cream-coloured microcrystalline powder, which could not be satisfactorily recrystallised from any solvent (wt. 1·98 g.). It was, therefore, purified for analysis by sublimation in a high vacuum at 220°. Samples of puberulic acid prepared by hydrolysis of the diacetyl compound, or by direct sublimation of the ether extract from *P. puberulum*, were identical, since they were cream-coloured, melted at 316° with decomposition and gave rise to the same diacetyl compound. Puberulic acid is only slightly soluble in water. On the addition of a slight

excess of $FeCl_3$ solution to a dilute alcoholic solution of the acid a deep reddish-brown colour is obtained. When the test is made by adding very dilute $FeCl_3$ solution to an alcoholic solution of the acid a yellow-brown colour is first obtained, followed by a distinct amethyst shade as further additions are made, and finally by the deep reddish-brown colour referred to above. Puberulic acid contains no nitrogen and no methoxyl groups. Micro-analysis (Schoeller): C, 48·60 %, 48·48 %; H, 3·16 %, 3·09 %. ($C_8H_6O_6$ requires C, 48·48 %; H, 3·05 %.)

Equivalent by titration, 95·4; for a dibasic acid, $C_8H_6O_6$, the calculated equivalent is 99.

The acid $C_8H_4O_6$ was prepared from the crude sodium salt separating from the diluted acetylation mixture of the mixed acids (p. 443). The crude sodium salt was dissolved in methyl alcohol, two volumes of ether were added to precipitate a dark-coloured amorphous material, and the product, after removal of solvent, was recrystallised from ethyl alcohol. It was finally obtained in beautiful orange needles containing 10·12 % Na. ($C_8H_3O_6.Na$ requires Na 10·55 %.)

The pure sodium salt was dissolved in hot water and acidified with HCl. On cooling, the free acid separated in shining yellow plates, M.P. 296° (decomp.). An alcoholic solution of the acid gives with $FeCl_3$ a final colour indistinguishable from that given by puberulic acid. Micro-analysis (Schoeller) (two different samples of the free acid): C, 48·75 %, 48·92 %; 48·58 %, 48·52 %; H, 2·30 %, 2·39 %; 2·09 %, 2·08 %; mol. wt., 217, 187. ($C_8H_4O_6$ requires C, 48·97 %; H, 2·06 %; mol. wt., 196.)

Although it seems probable that puberulic acid and the acid $C_8H_4O_6$ are intimately related, the former being probably a reduced form of the latter, we have not yet succeeded in transforming either acid into the other.

Diacetylpuberulic acid, $C_8H_4O_4(O.CO.CH_3)_2$, was prepared from samples of puberulic acid obtained from both *P. puberulum* and *P. aurantio-virens*, by acetylation with sodium acetate and acetic anhydride as previously described. In each case the product was obtained as well-defined colourless prisms, melting at 212° and having the same mixed melting-point. The acetyl compound is readily soluble in aqueous sodium acetate solution and in alcoholic solution gives no colour with $FeCl_3$.

Micro-analysis (Schoeller): C, 51·08 %, 51·24 %; H, 3·77 %, 3·72 %. Mol. wt. by Rast method, 216, 232. ($C_{12}H_{10}O_8$ requires C, 51·05 %; H, 3·57; mol. wt., 282.)

0·0812 g. dissolved in 10 cc. alcohol, required 5·72 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 142. (Theoretical for $C_{12}H_{10}O_8$ titrating as a dibasic acid, 141.) 0·4030 g. when hydrolysed in an atmosphere of nitrogen with *N*/5 NaOH yielded acetic acid equivalent to 13·91 cc. of *N*/5 NaOH (calc. for 2 acetyl groups, 14·29 cc.).

Dimethylpuberulic acid, $C_8H_4O_4(OCH_3)_2$. 1 g. of puberulic acid and 10 cc. (a large excess) of dimethyl sulphate were shaken vigorously and 10 %

aqueous NaOH were added continuously to maintain an alkaline reaction. When most of the dimethyl sulphate had disappeared an excess of NaOH was added, the mixture was boiled for some time, cooled, acidified, extracted with ether, and the ethereal solution evaporated to dryness. The residual solid, the yield of which was small, was crystallised from a mixture of methyl alcohol and water, and finally purified for analysis by sublimation in a high vacuum. The sublimate, which was colourless, melted at 271–272°, but gave a weak reddish-brown colour in alcoholic solution with $FeCl_3$.

Micro-analysis (Schoeller): C, 53·10 %; H, 4·68 %; OCH_3, 25·8 %. ($C_8H_4O_4(OCH_3)_2$ requires C, 53·08 %; H, 4·45 %; OCH_3, 27·4 %.)

Equivalent by titration, 116. (Theoretical for $C_8H_4O_4(OCH_3)_2$, titrating as a dibasic acid, 113.)

A sample of dimethylpuberulic acid, made by hydrolysing tetramethylpuberulic acid (p. 446) with boiling aqueous *N* sodium hydroxide, gave no colour with $FeCl_3$ in alcoholic solution. This sample contained 26·67 % OCH_3. It thus appears that the colour given with $FeCl_3$ by the sample prepared by methylation with methyl sulphate and sodium hydroxide is due to the presence of traces of partially methylated material and indicates that at least one hydroxyl group in puberulic acid is difficult to methylate under these conditions.

Dimethyldiacetylpuberulic acid, $C_8H_2O_2(OCH_3)_2(O.CO.CH_3)_2$. When diacetylpuberulic acid was treated with an excess of diazomethane in ether, a syrup was obtained which could not be crystallised. When, however, diacetylpuberulic acid was treated with just sufficient diazomethane in ether to dissolve it, at which point the initial violent evolution of nitrogen ceased, a crystalline product was obtained. 0·7 g. of diacetylpuberulic acid, treated in this way, gave, after evaporation in air of most of the ether, a mass of needles which separated from the ether mother-liquors. The product on crystallisation from methyl alcohol gave 0·45 g. of colourless needles, melting sharply at 102° and quickly turning red on exposure to light. The crystals were neutral in reaction and gave no colour with $FeCl_3$ in alcoholic solution.

Micro-analysis (Schoeller): C, 54·28 %; H, 4·51 %; OCH_3, 19·94 %; mol. wt., 286. ($C_8H_2O_2(OCH_3)_2(O.CO.CH_3)_2$ requires C, 54·18 %; H, 4·55 %; OCH_3, 20·01 %; mol. wt., 310.)

0·4261 g. when hydrolysed in nitrogen with *N* NaOH yielded acetic acid equivalent to 27·54 cc. of *N*/10 NaOH, indicating an acetyl content of 27·8 % (calc. 27·75 %).

0·1047 g. of the substance was hydrolysed by boiling for 2½ hours in an atmosphere of nitrogen with a measured excess (25 cc.) of *N*/5 NaOH. The mixture was cooled and the residual NaOH estimated. It was thus shown that an increase in acidity corresponding to 13·44 cc. *N*/10 acid had been produced, compared with the theoretical figure of 13·51 cc. required for the liberation of 4 acidic groups. It is thus obvious that, since two molecules of acetic acid are produced on hydrolysis, two other acid reacting groups have been unmasked during hydrolysis.

Monomethylpuberulic acid, $C_8H_5O_5(OCH_3)$. The alkaline hydrolysis mixture from dimethyldiacetylpuberulic acid yielded, when acidified, a product crystallising readily from water in the form of glistening plates, M.P. 272–276°, which on further heating decomposed slowly at 320–330°. It gave a brown colour in alcoholic solution with ferric chloride.

Micro-analysis (Schoeller): C, 51·23, 51·19 %; H, 3·81, 3·82 %; OCH_3, 14·59, 14·43 %. ($C_8H_5O_5(OCH_3)$ requires C, 50·93 %; H, 3·80 %; OCH_3, 14·63 %.)

Equivalent by titration, 105·1. (Theoretical for $C_8H_5O_5(OCH_3)$ titrating as a dibasic acid, 106.)

Tetramethyl derivative, $C_8H_2O_2(OCH_3)_4$. 1·5 g. of puberulic acid were suspended in dry ether and treated with a large excess of an ethereal solution of diazomethane. A vigorous effervescence took place. When this had ceased the solution was filtered from a small amount of insoluble material (0·08 g.) and evaporated to about 50 cc. On chilling the solution the product separated in clusters of pale yellow needles, weight 0·67 g.; M.P. 112–113°. The residue consisted of a yellow oil.

The tetramethyl derivative, surprisingly enough, is readily soluble in water, and in most other solvents, is neutral in reaction, gives no colour in alcoholic solution with $FeCl_3$, and contains no nitrogen.

Micro-analysis (Schoeller): C, 56·64, 56·64 %; H, 5·59, 5·62 %; OCH_3, 48·18, 48·20 %. ($C_8H_2O_2(OCH_3)_4$ requires C, 56·67 %; H, 5·55 %; OCH_3, 48·83 %.)

Molecular weight estimations (calc. 254) by the Rast method for which we are indebted to Prof. C. R. Harington gave abnormal results. They varied from an initial value of 474 to a final and fairly steady value of 275 after repeated remelting and cooling, in the course of which there was no obvious gross decomposition.

The results obtained may now be reviewed in the light of their bearing on the constitution of puberulic acid.

(1) Puberulic acid, which is cream-coloured, has the empirical formula $C_8H_6O_6$, titrates sharply to phenolphthalein as a dibasic acid, and gives a purple-brown colour with $FeCl_3$.

(2) Puberulic acid gives with diazomethane a tetramethyl derivative, $C_8H_2O_2(OCH_3)_4$, which is pale yellow-coloured, readily soluble in cold water, neutral in reaction, and gives no colour with $FeCl_3$. Puberulic acid therefore contains four OH groups.

(3) Puberulic acid gives a colourless diacetyl compound, $C_8H_4O_4(O.CO.CH_3)_2$, with acetic anhydride and sodium acetate, and a colourless dimethyl derivative, $C_8H_4O_4(OCH_3)_2$, with dimethyl sulphate and sodium hydroxide. Both the diacetyl and dimethyl derivatives titrate sharply to phenolphthalein as dibasic acids. Neither diacetyl- nor dimethyl-puberulic acid gives a colour with $FeCl_3$.

(4) Diacetylpuberulic acid, $C_8H_4O_4(O.CO.CH_3)_2$, gives with diazomethane dimethyldiacetylpuberulic acid in colourless crystals turning pink on exposure

to light. This compound, $C_8H_2O_2(OCH_3)_2(O.CO.CH_3)_2$, is neutral in reaction, and gives no colour with $FeCl_3$. On gentle hydrolysis it loses both acetyl groups and one OCH_3 group to give monomethylpuberulic acid, $C_8H_5O_5(OCH_3)$, a substance titrating sharply to phenolphthalein as a dibasic acid, and giving a brown colour with $FeCl_3$.

It is thus abundantly clear that puberulic acid contains four OH groups, two of which are probably phenolic in nature, since puberulic acid gives a strong colour with $FeCl_3$, and the diacetyl and dimethyl derivatives give no colour with $FeCl_3$. The nature of the two remaining OH groups, which confer strong acidic properties on puberulic acid, but give no colour with $FeCl_3$, is not quite certain. The most obvious explanation is that they are present in carboxyl groups in which case the formula for puberulic acid becomes $C_6H_2(OH)_2(COOH)_2$ and the acid would probably be a dihydroxybenzenedicarboxylic acid. This explanation agrees with most of the facts presented. Thus the diacetyl and dimethyl compounds would be diacetyl- and dimethoxybenzenedicarboxylic acids, while the diacetyldimethyl- and tetramethylcompounds would be the dimethyl esters of diacetyl- and dimethoxy-benzenedicarboxylic acids respectively. The most serious objection to this hypothesis arises when puberulic acid and its derivatives are compared with the known dihydroxybenzenedicarboxylic acids, eleven of which are theoretically possible.

These may be grouped as derivatives of phthalic, *iso*phthalic and terephthalic acids.

A. Dihydroxyphthalic acids, COOH : COOH : 1 : 2; (i) 3 : 4-dihydroxy; (ii) 3 : 5-dihydroxy; (iii) 3 : 6-dihydroxy; (iv) 4 : 5-dihydroxy. Of these (i), (iii) and (iv) cannot be identical with puberulic acid, since they all readily form anhydrides, the colours they give with $FeCl_3$ are deep blue, blue-violet and emerald green respectively, and they melt at 210–212°, 219–220° and 183–185° respectively. Acid (ii) has not been synthesised. β-Resodicarboxylic acid was formerly thought to have this constitution, but is now known to have formula (x) [Brunner, Madersbacher and Goritschan, 1928]. Since, however, acid (ii) would almost certainly readily form an anhydride, it is improbable that puberulic acid has this constitution.

B. Dihydroxy*iso*phthalic acids, COOH : COOH : 1 : 3; (v) 2 : 4-dihydroxy; (vi) 2 : 5-dihydroxy; (vii) 4 : 5-dihydroxy; (viii) 4 : 6-dihydroxy.

Acid (v), α-resodicarboxylic acid, has a recorded melting-point (305°, 312°) sufficiently near to that of puberulic acid (316°) to suggest a possible identity. A quantity of acid (v) was therefore synthesised from β-resorcylic acid by the method of Errera [1899]. It melted at 309°, a sublimed sample of puberulic acid melted at 316°, and a mixture of the two at 292° under identical conditions of heating. This fact, together with a totally different colour reaction with $FeCl_3$—wine-red against the purple-brown given by puberulic acid—definitely exclude (v) as the constitution of puberulic acid. Further, acid (v), in contrast with puberulic acid, does not give an acetyl derivative on heating with acetic anhydride and sodium acetate.

Acid (vi) has not been previously synthesised. Its synthesis by two different methods is described in the Appendix. The acid prepared by either method gave a blue-violet colour with $FeCl_3$ and melted at 248–250°, the dimethyl ether melted at 172°. These melting-points are very different from those of puberulic acid and its dimethyl ether, 316°, and 272°. Acid (vi) is thus definitely not identical with puberulic acid.

The free acid (vii) has not been described, but it can scarcely be identical with puberulic acid since its dimethyl derivative melts at 245–246° [Tiemann and Mendelsohn, 1877] while dimethylpuberulic acid melts at 271–272°, and, since acid (vii) is a catechol derivative, it would probably give a blue or green colour with $FeCl_3$.

The free acid (viii) has not been described previously, although a dimethyl ether, M.P. 266°, prepared by Eykman [1905], was thought by its discoverer to be 4 : 6-dimethoxybenzene-1 : 3-dicarboxylic acid but is now shown (see Appendix) to be identical with α-resodicarboxylic acid. We prepared a quantity of this material by the method given by the above author and, by demethylation with concentrated HI, obtained the parent dihydroxy-acid. The latter melts at 311–312° but is not identical with puberulic acid since the acids have a mixed M.P. of 288°, it gives a wine-red colour with $FeCl_3$, and does not give an acetyl derivative on heating with acetic anhydride and sodium acetate.

C. Dihydroxyterephthalic acids, COOH : COOH : 1 : 4; (ix) 2 : 3-dihydroxy; (x) 2 : 6-dihydroxy; (xi) 2 : 5-dihydroxy.

Acid (ix), described by Kawai [1926], cannot be identical with puberulic acid. It melts at 290°, gives a deep indigo-blue colour with $FeCl_3$, and its dimethyl ether melts at 213–215°.

Acid (x), β-resodicarboxylic acid, was formerly thought to have formula (ii), but has now been shown [Brunner, Madersbacher and Goritschan, 1928] to have the formula indicated. A specimen of this acid was made and compared with puberulic acid. The two acids are definitely not identical. Acid (x) melted at 284°, and gave a blue-violet colour with $FeCl_3$ in alcohol. Further, its dimethyl ether was found to melt at 301° (Wedekind and Fleischer [1924] give 295°) and its diacetyl derivative is recorded as melting at 198° [Brunner, Madersbacher and Goritschan, 1928].

Acid (xi), which is yellow in colour, is well known. A specimen of the acid made by Herrmann's method [1882] melted at 346°, and gave a pure blue colour with $FeCl_3$. Further, since its diacetyl derivative was found not to melt, even at 360° (Thiele and Gunther [1906] state that it does not melt up to 260°), there can be no doubt that puberulic acid has not the formula of acid (xi).

If the evidence afforded by the dihydroxybenzenedicarboxylic acids which we have prepared, and if the chemical constitutions and physical characteristics of the other dihydroxybenzenedicarboxylic acids described in the literature are accepted as correct, the possibility of puberulic acid being a dihydroxybenzenedicarboxylic acid is practically excluded, though there is, of course, the remote possibility that it may be either 2 : 4-dihydroxybenzene-1 : 3-di-

carboxylic acid or 4 : 6-dihydroxybenzene-1 : 3-dicarboxylic acid, depending on which of these formulae is finally assigned to α-resodicarboxylic acid. Even this possibility seems to be ruled out by the yellow colour of the tetramethyl derivative and its ready solubility in water.

In an attempt to demonstrate the presence of aldehydic or ketonic groups the following qualitative tests were carried out in each case on cold neutral aqueous solutions of the ammonium salts of the free acid, the monomethyl and diacetyl derivatives, and on cold aqueous solutions of the tetramethyl and the diacetyldimethyl derivatives.

(i) Schiff's reagent gave a negative in all cases.

(ii) 2 : 4-Dinitrophenylhydrazine gave no precipitate in any case.

(iii) Cold ammoniacal silver nitrate gave an immediate strong reduction with puberulic acid. There was no immediate reduction with any of the derivatives, though the diacetyl derivative gave a partial reduction after standing for about an hour and the diacetyldimethyl and monomethyl derivatives after standing overnight. On the other hand, tetramethylpuberulic acid showed no sign of reduction, even after standing for 2 days.

(iv) Cold neutral $KMnO_4$. Immediate strong reduction was given by puberulic acid and monomethylpuberulic acid. A moderately quick though not immediate reduction was given by diacetylpuberulic acid, and a slow reduction on standing was given by the diacetyldimethyl and tetramethyl derivatives.

Tests (i) and (ii) give no support to the view that puberulic acid, or any of its derivatives, contains either an aldehydic or ketonic grouping. On the other hand, test (iii) might be taken to indicate the presence of an aldehydic grouping in puberulic acid. In this connection it is of interest to note that while the so-called 2 : 4-dihydroxybenzene-1 : 3-dicarboxylic acid (acid v) and 2 : 6-dihydroxybenzene-1 : 4-dicarboxylic acid (acid x) give no immediate reduction with either ammoniacal silver or cold neutral $KMnO_4$ solution, 2 : 5-dihydroxybenzene-1 : 3-dicarboxylic acid (acid vi) and 2 : 5-dihydroxybenzene-1 : 4-dicarboxylic acid (acid xi) give immediate strong reduction of both reagents.

We therefore propose to leave the question of the exact determination of the constitution of puberulic acid to a future communication, in the opinion that, in spite of resemblances to certain of the dihydroxybenzenedicarboxylic acids, it is improbable that puberulic acid is a compound of this type.

Since there are good *a priori* reasons for assuming that puberulic acid $C_8H_6O_6$ and the acid $C_8H_4O_6$ are closely related, *e.g.* their formulae, their colour reactions with $FeCl_3$ and the fact that both acids are present in the same metabolism solution, it is tempting to speculate on the possibility of these two acids forming an oxidation-reduction system for the utilisation of atmospheric oxygen by the moulds which produce them—*P. puberulum* and *P. aurantiovirens*. This aspect of the question we propose to develop also in a future communication.

SUMMARY.

The production is described of two new mould metabolic products, puberulic acid $C_8H_6O_6$ and an acid $C_8H_4O_6$, in synthetic solutions containing glucose as sole source of carbon. Each of these acids is produced by *P. puberulum* Bainier and *P. aurantio-virens* Biourge. Puberulic acid and its monomethyl, dimethyl and diacetyl derivatives are dibasic acids, whereas the tetramethyl and diacetyldimethyl compounds are neutral substances. The possibility of puberulic acid being a dihydroxybenzenedicarboxylic acid is considered, but it is thought that this is improbable. It is suggested that puberulic acid $C_8H_6O_6$ and the acid $C_8H_4O_6$ may form an oxidation-reduction system.

We desire to express our thanks to Prof. Robert Robinson for much kindly advice and helpful criticism.

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APPENDIX ON CERTAIN DIHYDROXYBENZENEDICARBOXYLIC ACIDS.

In the course of work described above it became necessary to prepare certain new dihydroxybenzenedicarboxylic acids, and also certain derivatives, not previously described, of dihydroxybenzenedicarboxylic acids already appearing in the literature. An account of this work follows.

Synthesis of 2 : 5-dihydroxybenzene-1 : 3-dicarboxylic acid.

This acid, which has not previously been described, was synthesised from 2 : 5-dihydroxy-1 : 3-dimethylbenzene [*cf.* Bamberger and Rising, 1901] by conversion (*a*) into the potassium sulphuric ester, or (*b*) into the dimethyl ether followed by oxidation and hydrolysis.

(*a*) *2 : 5-Dihydroxy-1 : 3-dimethylphenyl dipotassium disulphate.* 2 g. of 2 : 5-dihydroxy-1 : 3-dimethylbenzene were mixed with 4·6 g. of *N*-pyridinium sulphonic acid and heated in an oil-bath at 100°. The mixture slowly began to melt. A few drops of pyridine were then added. After a few minutes the mixture was dissolved in water and extracted with ether. 2·5 g. of potassium hydroxide (*i.e.* a slight excess) in water were added to the ether-extracted solution which was evaporated *in vacuo* to crystallisation. Yield of crystalline product, 3·2 g.

2 : 5-Dihydroxybenzene-1 : 3-dicarboxylic acid. 3·2 g. of the above compound were dissolved in about 300 cc. of water and oxidised with 5·5 g. of potassium permanganate at 100°. The mixture was then rendered weakly acid with sulphuric acid and hydrolysed for half an hour at 100°. On keeping, a brownish crystalline deposit was obtained, which was extracted with ether, filtered

from the insoluble colouring matter, and the fluorescent ether solution was evaporated, giving a mass of crystalline needles. The acid was further purified by neutralisation with sodium hydroxide, filtration from a small amount of insoluble matter, and reprecipitation by addition of a slight excess of sulphuric acid. It crystallised in beautiful long colourless needles with $1H_2O$, M.P. 248–250°, giving a blue-violet colour with $FeCl_3$. 0·0905 g. dried at 100° to constant weight (2 hours) lost 0·0068 g., *i.e.* 7·52 %. Theoretical for $C_8H_6O_6.H_2O$, 8·33 %. 0·0837 g. (anhydrous) required 8·54 cc. *N*/10 NaOH giving an equivalent of 98 (calc. 99).

Micro-analysis (Schoeller) on the material dried at 100° gave the following results: C, 48·61 %; H, 3·10 %. (Theoretical for $C_8H_6O_6$, C, 48·48 %; H, 3·05 %.)

(*b*) *2 : 5-Dimethoxy-1 : 3-dimethylbenzene.* 13·8 g. of crude 2 : 5-dihydroxy-1 : 3-dimethylbenzene were shaken with 40 cc. of 20 % sodium hydroxide and 26 g. of dimethyl sulphate. After some time a further addition of alkali and dimethyl sulphate was made, and the mixture was heated for a few minutes under reflux, the reaction being maintained permanently alkaline. The resulting product was distilled in steam, extracted from the distillate with ether, dried with calcium chloride and distilled *in vacuo*. Practically the whole of it boiled at 117°/25 mm. Yield 10·6 g.

2 : 5-Dimethoxybenzene-1 : 3-dicarboxylic acid. To 38 g. of $KMnO_4$ in 750 cc. of water were added 10 g. of the dimethoxy compound, and the mixture was heated in a water-bath under reflux for several hours. The hydrated manganese dioxide was then removed by filtration and the filtrate acidified with sulphuric acid and evaporated. The residue was extracted with ether and the product was recrystallised three times from water, from which it separated in silky needles melting at 172°. The yield was only small as the loss on oxidation was considerable. 0·0809 g. required 7·17 cc. *N*/10 NaOH giving an equivalent of 112·9. (Theoretical for $C_6H_2(COOH)_2(OCH_3)_2$, 113.) In a Zeisel estimation 0·0912 g. gave 0·1872 g. of silver iodide. OCH_3, 27·1 %. (Theoretical for $C_6H_2(COOH)_2(OCH_3)_2$, 27·4 %.) The acid gave no colour with ferric chloride.

2 : 5-Dihydroxybenzene-1 : 3-dicarboxylic acid. The residue from the Zeisel estimation gave well formed needles on cooling. These were separated by filtration from the hydriodic acid, washed and recrystallised from water. The product agreed in M.P., colour with ferric chloride, and mixed M.P. with the 2 : 5-dihydroxybenzene-1 : 3-dicarboxylic acid synthesised by method (*a*).

Preparation of 4 : 6-dihydroxybenzene-1 : 3-dicarboxylic acid.

The compound described by Eykman [1905] as 4 : 6-dimethoxybenzene-1 : 3-dicarboxylic acid was prepared in quantity by his method and had M.P. 276° (decomp.) (Eykman, 266°). It crystallised in colourless needles, gave no colour with $FeCl_3$, and gave the following results on analysis (Schoeller), C, 52·89 %; H, 4·45 %; OCH_3, 26·8 %. (Theoretical for $C_6H_2(OCH_3)_2(COOH)_2$, C, 53·08 %; H, 4·45 %; OCH_3, 27·4 %.)

0·0523 g. required 4·645 cc. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 112·6. (Theoretical for $C_6H_2(OCH_3)_2(COOH)_2$ titrating as a dibasic acid, 113.) The *dimethyl ester* (OCH_3, 48·7 %; calc., 48·8 %), prepared with diazomethane in ethereal solution, formed glistening plates, M.P. 151–153° after softening at 150°.

4 : 6-Dihydroxybenzene-1 : 3-dicarboxylic acid. 5 g. of the dimethoxy-acid were de-methylated with 50 cc. of HI (sp. gr. 1·7) in the Zeisel apparatus. The mixture was heated to 120–130° for 2½ hours, when no further reaction was obtained from the exit gas with alcoholic silver nitrate, cooled and allowed to stand. The solid material separating was filtered through a sintered glass funnel washed with water and dried. It melted at 311–312°, with decomposition, and gave a wine-red colour in alcoholic solution with ferric chloride.

Micro-analysis (Schoeller) gave the following results: C, 48·53 %; H, 3·09 %. (Theoretical for $C_8H_6O_6$, C, 48·48 %; H, 3·05 %.)

0·1000 g. required 10·12 cc. of *N*/10 sodium hydroxide giving an equivalent of 98·8. (Theoretical for $C_8H_6O_6$ titrating as a dibasic acid, 99.) It was found impossible to acetylate this acid by heating with acetic anhydride and sodium acetate. The only product which could be recovered (0·62 g. from 1 g. of the dihydroxy-acid) melted at 302°, gave a deep red colour with $FeCl_3$, and contained no free acidic groups.

α-Resodicarboxylic acid.

α-Resodicarboxylic acid, which is thought to have the constitution 2 : 4-dihydroxybenzene-1 : 3-dicarboxylic acid, was synthesised from β-resorcylic acid by the method of Errera [1899].

The colourless product, melting at 309° with decomposition, gave a wine-red colour with $FeCl_3$, and did not give an acetyl compound on heating with acetic anhydride and sodium acetate.

Micro-analysis (Schoeller) gave the following results: C, 48·27 %; H, 3·09 %. (Theoretical for $C_8H_6O_8$, C, 48·48 %; H, 3·05 %.)

0·0472 g. required 4·72 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 100. (Theoretical for $C_8H_6O_6$ titrating as a dibasic acid, 99.)

Direct comparison of α-resodicarboxylic acid with the acid prepared by demethylation of Eykman's dimethoxybenzenedicarboxylic acid (see above) showed that they are probably the same substance. Their melting-points are 309°, and 311–312° respectively, while a mixture of the two melted at 309–310°. They each give the same wine-red colour with $FeCl_3$, and neither of them gives an acetyl derivative on heating with acetic anhydride and sodium acetate.

Dimethyl-α-resodicarboxylic acid. α-Resodicarboxylic acid was methylated three times with methyl sulphate and excess of sodium hydroxide (20 %). The methylation mixture was allowed to warm up spontaneously and then boiled for a short time, cooled and finally acidified with H_2SO_4. This protracted methylation is necessary since α-resodicarboxylic acid is difficult to methylate. The solid separating was washed and dried.

Micro-analysis (Schoeller) gave the following results: C, 53·16 %; H, 4·61 %; OCH_3, 26·9 %. (Theoretical for $C_6H_2(OCH_3)_2(COOH)_2$, C, 53·08 %; H, 4·45 %; OCH_3, 27·4 %.)

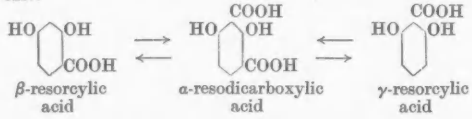
0·0506 g. required 4·54 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 111·7. (Theoretical for $C_6H_2(OCH_3)_2(COOH)_2$ titrating as a dibasic acid, 113.)

The product gave no colour with $FeCl_3$ and had a melting-point of 275° (decomp.). A mixed melting-point with Eykman's dimethoxybenzenedicarboxylic acid was also 275°.

Treatment with diazomethane in ether gave a dimethyl ester identical with that similarly prepared from Eykman's acid. The same dimethyl ester is given, though in smaller yield since methylation even with diazomethane is not complete, by methylating α-resodicarboxylic acid directly with diazomethane.

It is thus obvious that α-resodicarboxylic acid and the dihydroxybenzenedicarboxylic acid prepared from Eykman's 4 : 6-dimethoxybenzene-1 : 3-dicarboxylic acid are one and the same substance. Since α-resodicarboxylic acid is thought to have the constitution 2 : 4-dihydroxybenzene-1 : 3 dicarboxylic acid, and since Eykman's product is thought to be the dimethyl derivative of 4 : 6-dihydroxybenzene-1 : 3-dicarboxylic acid, it is obvious that the formula for one or other of these acids must be incorrect.

The formula for α-resodicarboxylic acid and the evidence supporting this formula were presented by Waitz [1911]. Waitz showed that when either β-resorcylic acid or γ-resorcylic acid is heated with ammonium carbonate and water at 130° for 20 hours, α-resodicarboxylic acid is produced in each case.



Waitz points out that this proof of constitution is open to the objection that one or other of the substituent groups may have "wandered," but supports his evidence by showing that when α-resodicarboxylic acid is heated in water for 48 hours at 100° in a sealed tube, a mixture of β- and γ-resorcylic acids is formed. Accepting this evidence therefore, and omitting any possibility of the "wandering" of substituent groups, there appears to be no alternative to the acceptance of the statement that α-resodicarboxylic acid is 2 : 4-dihydroxybenzene-1 : 3-dicarboxylic acid.

The evidence for the constitution of Eykman's dimethoxybenzenedicarboxylic acid, thought by him to be 4 : 6-dimethoxybenzene-1 : 3-dicarboxylic acid, obviously depends on the orientation of the groups in the parent dihydroxydiacetylbenzene or "resodiacetophenone" which was prepared by heating resorcinol diacetate with $ZnCl_2$. Eykman prepared a large number of mixed ethers by the action of alkyl iodides or bromides on the potassium salt of his dihydroxydiacetylbenzene. Since mixed ethers of the type $C_6H_2(COCH_3)_2(OR_1)(OR_2)$ are identical, independently of the order of the introduction of the groups R_1 and R_2, the parent acetyl compound must be

4 : 6-dihydroxy-1 : 3-diacetylbenzene and hence the acid resulting from the oxidation of the dimethyl ether must be 4 : 6-dimethoxybenzene-1 : 3-dicarboxylic acid.

Further work is contemplated to settle which of these constitutions is correct.

Summary.

The synthesis by two different methods of 2:5-dihydroxybenzene-1:3-dicarboxylic acid is described. It is proved that α-resodicarboxylic acid, at present thought to be 2:4-dihydroxybenzene-1:3-dicarboxylic acid, is identical with the dihydroxybenzenedicarboxylic acid obtained by demethylation of the substance described by Eykman as 4:6-dimethoxybenzene-1:3-dicarboxylic acid. This follows since the dihydroxy-acids, the dimethyl ethers and the dimethyl ether dimethyl esters are identical in each case.

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LIV. A p_H CHART.

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(*Received January 22nd, 1932.*)

This chart[1] was originally constructed to facilitate calculations on the measurement of p_H by the hydrogen electrode method. It gives, graphically, the solution to the equation

$$p_H = \frac{\text{E.M.F. of chain } (V) - \text{E.M.F. of calomel cell } (v)}{0{\cdot}000198322\ T} \qquad \ldots\ldots(1),$$

or, more generally,

$$p_H = \frac{E}{2{\cdot}303\frac{RT}{F}} \qquad \ldots\ldots(2),$$

from p_H 1·5 to 12·0 over the usual laboratory range of temperature, *viz.* 18° to 25°.

This graph has been found to be more convenient than tables such as those of Schmidt and Hoagland [1919], and of more general application than the graph of McClendon [1916].

Applications.

(1) *Hydrogen electrode.* From the potential of the chain as found on the potentiometer subtract the E.M.F. (corrected for temperature) of the calomel cell in use. Find this value, designated $V - v$, on the upper edge of one of the sections of the chart, and from it carry a line downwards parallel to the sloping lines till the required temperature line (marked in °C. at the end of each section) is met. From the point of intersection drop a vertical to the lower edge which is marked off in p_H units. It should be possible to read the results to 0·01 p_H unit.

(2) *Glass electrode.* The preceding remarks apply particularly to the hydrogen electrode but it will be remembered that equation (2) is of common occurrence in p_H formulae. The formula for a glass electrode of a character suitable for p_H determinations is

$$E = 2{\cdot}303\frac{RT}{F}(p_{H_1} - p_{H_2}) + E_g \qquad \ldots\ldots(3),$$

where E is the potential measured across the membrane, and E_g is the potential when the solutions on either side of the membrane are the same, *i.e.* when $p_{H_1} = p_{H_2}$.

[1] The chart is here reproduced upon a scale which is approximately two-thirds of the original.

From (3)

$$p_{H_1} - p_{H_2} = \frac{E - E_q}{2{\cdot}303\,\frac{RT}{F}} \qquad \ldots\ldots(4),$$

which is of the same form as equation (2). The value of $p_{H_1} - p_{H_2}$ can therefore be obtained from the graph. p_{H_2} (a standard buffer solution of known p_H is used) added to this gives then the value of p_{H_1}.

(3) *The quinhydrone electrode.* The graph is also applicable to this case. In the following chain

Au/quinhydrone sol. of known p_H/sat. KCl/sat. calomel cell,

$$p_H = \frac{E_a + e_q}{2{\cdot}303\,\frac{RT}{F}},$$

or

$$2{\cdot}303\,\frac{RT}{F}\,p_H = E_a + e_q,$$

where E_a is the E.M.F. of the chain, and e_q is a constant.

By using the graph in the opposite direction to that given under "hydrogen electrode" above, one can find a voltage V corresponding to the known p_H. Since the graph gives

$$V = 2{\cdot}303\,\frac{RT}{F}\,p_H,$$

then

$$V = E_a + e_q \text{ or } e_q = V - E_a.$$

Now in the following chain of E.M.F. E_x

Au/quinhydrone sol. of unknown p_H/sat. KCl/sat. calomel cell,

$$p_H = \frac{E_x + e_q}{2{\cdot}303\,\frac{RT}{F}}.$$

E_x is observed and e_q has been found above. By using their sum in the graph the p_H may be read off directly.

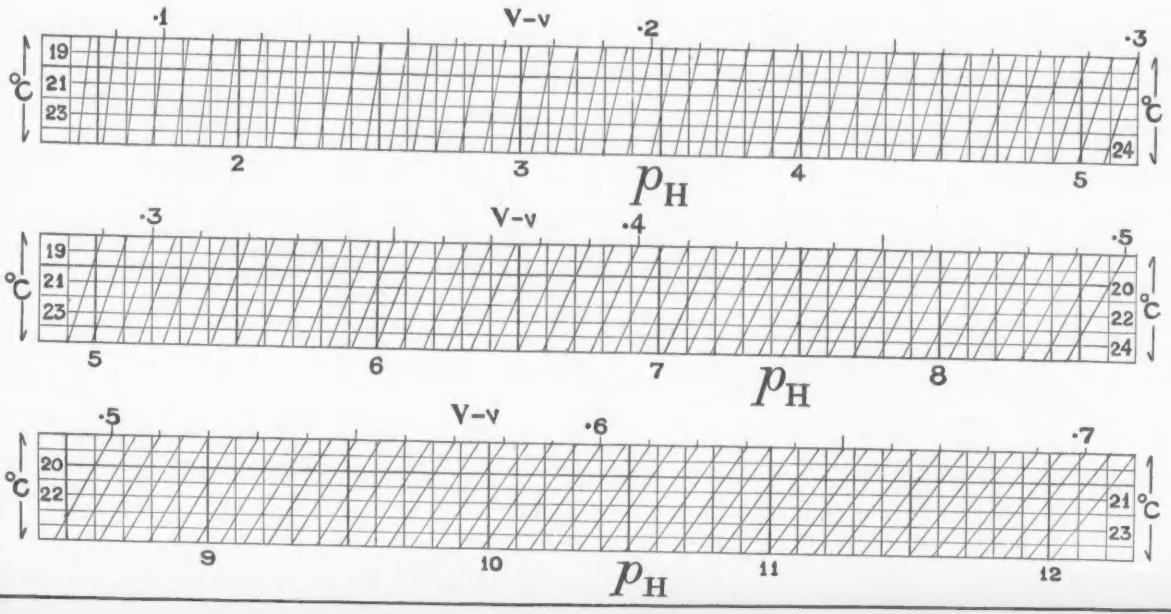
It may not be out of place here to make some remarks on the subject of the accuracy of the measurements as illustrated by this graph.

If p_H readings to $\pm$ 0·01 unit are required it is interesting to note the effect of the temperature. At p_H 2–3 it is necessary to know the temperature of the hydrogen half-cell only to the nearest degree. At p_H 6·5, 0·01 p_H unit corresponds to 0·43°. At p_H 10·5, 0·01 p_H unit corresponds to 0·27°, and therefore the temperature of the hydrogen half-cell must be known to 0·27°. The increase in the significance of T is shown on the graph by the greater angle that the sloping lines make with the vertical lines as the p_H figure increases.

It is customary in ordinary laboratory practice to neglect the effect of barometric pressure. The equation of the hydrogen electrode including the barometric correction, E_{bar}, is

$$p_H = \frac{E_0 + E_{bar} - E_{cal}}{0{\cdot}000198322\,T},$$

Chart to Solve $p_H = \frac{V-v}{0{\cdot}0001983ZT}$



where E_0 = observed potential; E_{cal} = potential of calomel electrode. Clark [1928] gives these figures:

At pressure 740 mm. Hg, 20°, $E_{bar} = + 0{\cdot}64$ millivolt.
At pressure 780 mm. Hg, 20°, $E_{bar} = - 0{\cdot}04$ millivolt.

From the graph it will be seen that 0·01 p_H unit is approximately equivalent to 0·5 millivolt. It is obvious that if p_H numbers correct to $\pm$ 0·01 are required it will be necessary in some experiments to allow for variations in barometric pressure.

It may be objected that this nomogram does not perform the subtraction $V - v$. The slide-rule of D. T. Harris and the nomogram of Grant [1930], which do perform this subtraction, do not claim to be readable, like the present chart, to 0·01 p_H. On the other hand, where this degree of accuracy is not required, these devices may be more convenient.

We wish to acknowledge our indebtedness to Prof. A. Hunter and Dr G. M. Wishart for much helpful criticism.

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LV. A NOTE ON THE DETERMINATION OF ERGOTHIONEINE IN BLOOD FILTRATES.

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(*Received February 5th, 1932.*)

Some points in connection with an article which has recently appeared by Salt [1931] on the ergothioneine content of human blood seem to call for comment. Methods for the determination of ergothioneine are still in the experimental stage and, until a method is available which has been proved to be really reliable, figures for the normal range of this compound in blood, and for its variation in pathological conditions, cannot be established. Under these circumstances any comments which may help to clarify the subject seem advisable at the present time.

A method for the determination of ergothioneine (then called thiasine) was described by Benedict, Newton and Behre [1926] which depended upon the amount of colour formed when sodium cyanide and the regular uric acid reagent [Benedict, 1922] were heated with the silver residue remaining after the indirect removal of uric acid by means of the silver lactate-acid sodium chloride treatment of whole blood-filtrates.

Subsequently a second method was described by Behre and Benedict [1929] which involved the determination of ergothioneine in the same silver residue but which utilised sodium hydroxide as alkali for the development of colour with the uric acid reagent, the amount of cyanide used being reduced to the minimum necessary for the solution of the silver residue. The colour reaction in this case takes place at room temperature. This method was believed to be more accurate than the earlier one because (1) the use of hydroxide (as alkali) instead of cyanide increases the specificity of the reaction for ergothioneine as compared with uric acid, and (2) lower ergothioneine figures were obtained from whole blood filtrates by the second method, while at the same time added ergothioneine was completely recovered. The fact that lower blood figures were found by the second method led us to assume that the silver residue contained some substance other than uric acid (and almost certainly not glutathione) which was capable of reacting in the presence of cyanide but not where sodium hydroxide was employed as the alkali. Although we still feel that the method just referred to is preferable to any of the others which we have tried, we are well aware that in its present form it has some disadvantages. The presence of even the small amount of cyanide used for the solution of the silver residue

reduces the depth of colour given by ergothioneine with sodium hydroxide, so that many of the blood filtrates give a final colour which is so light that the colorimetric reading must be made with the greatest care. It is also necessary to add an amount of cyanide to the ergothioneine standard exactly equal to the amount added to the silver residue[1]. No satisfactory standard other than a solution of pure ergothioneine has been found for use in this method. We are hoping shortly to be able to overcome these difficulties.

The procedure for the ergothioneine determination which has recently been described by Salt [1931] is essentially identical with our first method [Benedict, Newton and Behre, 1926] except that the determination is carried out on filtrates from the corpuscular residues obtained by the preparation of unlaked extracts according to Folin's directions [1930]. Salt [1931] does not specify his reason for using such filtrates (preparation of which involves two precipitations and a re-solution of one of these precipitates) in preference to using the filtrate from whole blood. Indeed, he presents figures which show that practically all the chromogenic substance is present in the corpuscles, so that double precipitation of the blood seems to have no special value. According to Salt's table, the amount of the chromogen present in the plasma fraction (the "unlaked extract") usually approaches zero and is never equivalent to more than 0·2 mg. of uric acid (per 100 cc.). This amount seems hardly great enough to justify the cumbersome procedure involved in the use of the corpuscular centrifugate. Nor is the amount of colour given by the unlaked plasma fraction great enough to warrant the assumption that it represents the unknown substance present in the silver residue from whole blood which, as we have stated, reacts in presence of cyanide and not with hydroxide. Taking this into consideration one is led to question whether the ergothioneine figures reported by Salt represent only ergothioneine or whether they may not also include a part of this unidentified, cyanide-reacting substance. The same doubt is cast upon other ergothioneine figures which have been based upon methods where cyanide is used [Rockwood, Turner and Pfiffner, 1929].

Salt's paper also includes a comparison of the blood-ergothioneine obtained after the use of different protein precipitants, taking the "direct" uric acid determination as an indication of the amount of ergothioneine present. According to his figures much less blood-ergothioneine was found in filtrates after precipitation with molybdic acid than after precipitation with either tungstic or tungstomolybdic acid. Figures for the recovery of ergothioneine added to blood are not given, but it is stated that added ergothioneine was quantitatively recovered from tungstomolybdic acid filtrates. The results with regard to molybdic acid are at variance with figures reported by Behre and Benedict [1929], and Benedict and Newton [1929], which showed that in the 15 bloods

[1] In the original directions for this method [Behre and Benedict, 1929], it was stated that 6 drops of the cyanide were to be added to the standard. Since more than 6 drops are sometimes necessary for the solution of the silver residue, this statement should be corrected to the effect that the same amount of cyanide is added to the standard as was added to the unknown.

examined ergothioneine added to blood was completely recovered after precipitation with molybdic acid.

Salt does, however, agree with our finding that tungstomolybdic acid causes no loss of ergothioneine. This protein precipitant is the one now employed in this laboratory preliminary to determination of any or all of the non-protein constituents of blood [Benedict, 1931].

With such uncertainty as to the methods at present available for the determination of ergothioneine in blood, it is obvious that no great reliance can be placed on the figures so far obtained. It is, however, interesting to find that Salt's figures corroborate our earlier findings [Benedict, Newton and Behre, 1926] to the effect that the ergothioneine content of diabetic blood tends to be higher than the normal. The average of Salt's figures for diabetic blood is nearly 3 mg. (38 %) above his average for normal blood. A quite similar relationship holds between Salt's average figure for ergothioneine in blood from diabetics as compared with blood from nephritics. It may also be noted that Salt's minimal and maximal figures for ergothioneine are definitely higher in diabetic than in normal blood.

A single determination on diabetic blood reported by Rockwood, Turner and Pfiffner [1929] also gave a slightly higher figure for ergothioneine than the normal figures reported by these workers. As Salt has pointed out, the range of values for ergothioneine in both normal and pathological blood appears to be so great that slight differences should not be too heavily stressed. That all the results on diabetic blood so far reported have tended in the same direction appears however to be of some significance. Apparently Salt was not familiar with the fact that we had already [Benedict, Newton and Behre, 1926] pointed out a definite tendency to higher ergothioneine figures for diabetic bloods. Had he known of our findings in this connection, he might have been less cautious in concluding with us that in diabetes there is tendency to an increased ergothioneine content of the blood.

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LVI. THE GLUTATHIONE CONTENT OF ANTI-ANAEMIC SUBSTANCES USED IN THE TREATMENT OF PERNICIOUS ANAEMIA.

By ROBERT FLEMING.

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(*Received February 11th, 1932.*)

It is generally agreed that the daily ingestion of fresh liver in the treatment of pernicious anaemia gives better results than those obtained from the use of various extracts prepared specifically by the drug industry for use in this disease. There is hardly any doubt, however, as to the fact that both supply in some measure a biological principle or principles necessary to the well-being of the patient. The difference therefore between the therapeutic value of fresh liver on the one hand and of the commercial preparations on the other is probably one of degree only. This difference might be accounted for by a loss or a modification of the active substance or substances during the process of preparation. It was considered of interest to examine some of these reputed blood-formation stimulants with respect to certain definite chemical substances which have been isolated from fresh liver. In the present communication we propose to deal only with glutathione. It is well known that glutathione occurs in appreciable quantities in liver, kidney, placenta, bone marrow and red blood corpuscles. We have also found this substance present in the gastric mucosa of the pig.

During a series of experiments on experimental anaemic conditions in animals we have had evidence that the glutathione content of various organs varies considerably from the supposed normal.

Several workers have determined the percentage of glutathione in fresh liver and have given figures ranging from 0·15 % to 0·40 %. These figures vary with the species and probably with different members of the same species. Further, it seems that all the glutathione is present in the reduced state.

As a type of commercial liver preparation we selected Heparglandol prepared by Hoffmann-La Roche, Bâle, who kindly furnished us with quantities sufficient for our experiments. This product is widely used in Switzerland in the treatment of anaemic conditions. It is easily soluble in hot water except for a small amount of fat which can be separated by filtration in the cold. A 10 % aqueous solution of Heparglandol gives a feeble nitroprusside reaction indicating the presence of an —SH group. If, however, a part of this solution

be gently boiled for a minute with an equal volume of a 5 % solution of sodium cyanide the resulting solution on cooling gives a vivid nitroprusside reaction. It is known that disulphides are reduced by cyanides but in the cold reduction is very slow and uncertainty sometimes exists as to when reduction is complete. We found that the boiling of a solution of a disulphide such as cystine or oxidised glutathione with sodium cyanide brings about a rapid and complete reduction when the requisite quantities are present. In a part of the reduced solution we estimated the quantity of —SH as reduced glutathione by a colorimetric method elsewhere described by us [1930].

The mean of many observations gave a glutathione content of 0·17 % while the amount of reduced glutathione before reduction was not more than 0·03 %. Ventriculin, a desiccated extract of gastric mucosa widely used in America and Europe in anaemic conditions, was also examined as to its glutathione content. Quantities of this product were kindly placed at our disposal by Messrs Parke Davis and Co. It is a greyish white granular substance stated to be insoluble. If, however, it is ground to a very fine powder and extracted with boiling water the resulting solution gives a positive nitroprusside reaction. Extraction with 10 % trichloroacetic acid presents no advantage over extraction with water. If the aqueous extract be reduced as described above in the case of Heparglandol the resulting solution gives a very strong colour reaction with nitroprusside.

It is very necessary to grind the Ventriculin as finely as possible to ensure maximum extraction of the glutathione. Even with this precaution we believe that a certain amount still remains adherent to the extracted residue. In fact, if the nitroprusside reaction were applied directly to this residue it frequently showed a strong reddish-violet colour. In order to obtain the strongest extract we found that it was best to add the sodium cyanide during the operation of extraction so that reduction and extraction were accomplished at the same time. The mean of many colorimetric estimations of glutathione in the reduced solution gave a content of 0·24 % while the amount in the original extract did not exceed 0·05 %.

The iodimetric estimation was not applicable to these products. The aqueous extract of Ventriculin before reduction, while showing but a feeble coloration with the nitroprusside reagent, yet required a large amount of iodine before oxidation was complete as shown by starch as inside indicator. It seems highly probable that the substance responsible for the nitroprusside reaction in these two preparations is glutathione. By the application of the dimethyl-*p*-phenylenediamine test for cystine we assured ourselves that cystine was absent in both cases.

Estimations of glutathione were also made in the fresh gastric mucosa of the pig. The mucosa was separated from the muscular layer with the aid of a pair of scissors. The tissue was finely minced and ground with a 10 % solution of trichloroacetic acid in presence of fine washed sand. The mixture was boiled and filtered. In one part the reduced glutathione was estimated

colorimetrically. Another portion was neutralised and reduced as before and the glutathione estimated. The mean of several observations gave a total value of 0·12 % of which 75 % was in the reduced state. We are ignorant of the proportions of this tissue used by the manufacturers in the production of Ventriculin.

It would seem from our observations that glutathione enters into the composition of anti-anaemic substances and that in the commercial products it appears mostly in the oxidised form. As to whether it has of itself any ameliorating effect in anaemic conditions we are as yet unable to say. Experiments on animals are being conducted with the object of elucidating this question.

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LVII. BACTERIAL METABOLISM.
II. GLUCOSE BREAKDOWN BY PNEUMOCOCCUS VARIANTS AND THE EFFECT OF PHOSPHATE THEREON.

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(*Received February 27th, 1932.*)

It was found that the lactic acid formed in cultures of haemolytic streptococci accounted for about 78 % of the glucose which had disappeared [Hewitt, 1932] and this proportion appeared not to vary under a variety of cultural conditions. In these respects the carbohydrate metabolism of haemolytic streptococci would seem to be less complex than that of many bacteria, since with many micro-organisms the products of glucose breakdown are very diverse and vary both qualitatively and quantitatively with the cultural conditions. The first object of this investigation, therefore, was to compare the carbohydrate metabolism of pneumococci with that of the closely related haemolytic streptococci.

With the haemolytic streptococcus variants investigated most glucose breakdown occurred in growing cultures of the matt virulent, less with the matt attenuated and least with the glossy variants. In each case more glucose breakdown occurred in the cultures showing heavier growth and it is uncertain whether heavier growth occurred owing to the greater ability of certain variants to break down glucose, or whether the amount of glucose breakdown is a measure of the amount of bacterial growth which is itself conditioned by other factors in the growth requirements of the different variants. In any event the fact remains that the differing antigenic structures of haemolytic streptococci and their differing virulence are associated with differences in the gross amount of chemical change produced in growing cultures. The appearance of 24-hour cultures in the medium used was sufficient to differentiate the variants on casual inspection.

Pneumococci also differ in antigenic behaviour, chemical structure and virulence and Finkle [1931] has described differences in the aerobic glycolysis effected by the different types of pneumococcus. Finkle worked with washed suspensions of bacteria in Ringer solution and estimated the glycolysis indirectly. The opportunity has now been taken of investigating the glucose breakdown in growing cultures of the different types of pneumococcus.

Since the growth requirements of pneumococci are, in general, similar to, although rather more rigorous than, those of haemolytic streptococci, it was

anticipated that the culture media described in the previous communication might prove suitable in the present experiments.

Analytical methods.

Glucose and lactic acid were determined by the methods previously described and the Bell-Doisy method was used for the determination of phosphorus. It was not considered necessary to treat the media with trichloroacetic acid before determining inorganic phosphate since so little material was precipitated by this reagent in the media employed.

Culture media.

The culture media used were constituted as follows:

	Medium no.			Phosphate-free
	M 6	M 7	M 8	M 9 and M 10
Glucose	10 g.	5 g.	10 g.	10 g.
$(NH_4)_2SO_4$	5 g.	5 g.	5 g.	5 g.
$NaHCO_3$	10 g.	Nil.	8 g.	8 g.
$Na_2HPO_4,12H_2O$	Nil.	8 g.	4 g.	Nil.
KCl	1 g.	1 g.	1 g.	1 g.
$MgSO_4,7H_2O$	0·01 g.	0·01 g.	0·01 g.	0·01 g.
$FeCl_3$	Nil.	Nil.	Nil.	Trace
0·02 % phenol red	3 cc.	Nil.	3 cc.	3 cc.
Tap-water	900 cc.	900 cc.	900 cc.	900 cc. (Distilled water)
Broth	100 cc.	100 cc.	100 cc.	100 cc. (Phosphate-free)

Before the addition of 100 cc. of broth to M 6 and M 8, CO_2 was bubbled through the medium until a reaction of about p_H 7·0 was reached. All the media were sterilised by filtration through Pasteur-Chamberland F candles and in the case of the bicarbonate media the media tubes were provided with cellophane caps secured by rubber bands over the cotton-wool plugs in order to minimise diffusion of carbon dioxide. The broth added to these media was a horse-flesh infusion containing 2 % of proteose peptone and had been sterilised by filtration and not by autoclaving or steaming.

Media M 9 and M 10 were similar to the others except that a trace of $FeCl_3$ was added and that inorganic phosphates were excluded; distilled water was used in place of tap-water, and the broth added had been freed from inorganic phosphates by adding 6 to 7 cc. of hot 10 % baryta to 100 cc. of broth, centrifuging, adding 2·5 to 3·0 cc. of *N* H_2SO_4 to remove barium, and again centrifuging. The clear supernatant fluid did not contain more than 3×10^{-6} g. per cc. of inorganic phosphorus.

Media 9 B, 9 C, *etc.* and 10 B, *etc.* were prepared by adding known volumes of a sterile 4 % sodium phosphate solution to M 9 and M 10. The glucose contents (Hagedorn-Jensen) of the media were M 6 1·035 %, M 7 0·548 %, M 8 1·055 %, M 9 1·020 %, and M 10 1·060 %.

Experimental.

The general experimental procedure was that previously described. In many experiments 50 cc. of culture medium contained in 7 × 1 in. tubes were used in place of 90 cc. batches in 7 × 1½ in. tubes. The inocula were each 0·2 cc. of a 24-hour broth culture of pneumococci in peptone-infusion broth. The strains of organisms used and their descriptions were kindly supplied by Dr J. M. Alston, Dr F. Griffith, and Dr G. F. Petrie. The organisms are designated as S (smooth) and R (rough) and by the number of the type; thus R II refers to the rough pneumococcus derived from Type II organisms.

Incubation of the cultures was conducted at 37° for 24 hours in all experiments and the analyses recorded were carried out on the filtrates of the cultures.

Lactic acid production under different conditions.

The data on lactic acid production from glucose are summarised in Table I. The mean value for the percentage yield of the lactic acid formed from the glucose broken down is 77·5 % and the extreme values are 72·2 and 83·5 %. The deviation from the mean value is small and within the limits of experimental error. In the case of haemolytic streptococci the mean value for the yield of lactic acid was 78 % and the limits were 68 % and 85 %. The figures for pneumococci and haemolytic streptococci are therefore, for practical purposes, identical. The yield of lactic acid appears not to vary over a range of variations of cultural conditions. Alterations were effected in the oxygen supply, in the inorganic phosphorus content (from 0·004 to 0·038 %), in the amount of sugar decomposed (from 0·215 to 0·992 %) and in the type and condition of the bacteria. All these appeared to be without sensible effect on the lactic acid yield.

Table I. *Lactic acid production from glucose by pneumococci.*

(Results are expressed in g. per 100 cc. of culture.)

Exp. no.	Type of organism	Medium	Inorganic P content of medium %	Conditions	Glucose disappeared	Lactic acid formed	Yield of lactic acid formed from glucose %
P 31	S I	M 6	0·004	Aerobic	0·215	0·164	76·3
P 36	R III	,,	0·004	,,	0·992	0·779	78·5
P 41	R III	,,	0·004	Anaerobic	0·872	0·685	78·6
P 42	S I	M 7	0·073	,,	0·248	0·195	78·6
P 43	S II	,,	0·073	,,	0·245	0·203	82·0
P 45	S III	,,	0·073	,,	0·225	0·174	77·3
P 46	R III	,,	0·073	,,	0·245	0·190	77·6
P 48	S I	,,	0·073	Aerobic	0·273	0·221	80·9
P 52	R III	,,	0·073	,,	0·303	0·253	83·5
P 53	S I	M 8	0·038	,,	0·545	0·422	77·5
P 54	S II	,,	0·038	,,	0·930	0·710	76·3
P 55	R II	,,	0·038	,,	0·845	0·610	72·2
P 56	S III	,,	0·038	,,	0·425	0·308	72·5
P 57	R III	,,	0·038	,,	0·980	0·765	78·1
P 80	R III	M 10 D	0·007	,,	0·844	0·645	76·4
P 81	R III	M 10 E	0·036	,,	0·914	0·674	73·7

Glucose breakdown by different types.

The ratio of lactic acid produced to glucose broken down is the same for the different forms of pneumococcus, as seen in Table I, but the actual amount of glucose broken down (or lactic acid formed) is different for the different types. In a typical experiment six 90 cc. tubes of bicarbonate medium M 6 were each inoculated with 0·5 cc. of a broth culture of a pneumococcus variant. After 24 hours' incubation there was very heavy growth in the R III culture, slight growth was visible in the S I culture and none of the others showed signs of growth. The R III culture was acid in reaction and effervescing freely (due to the liberation of CO_2 from the bicarbonate), whilst the others remained nearly neutral and showed no signs of liberation of gas bubbles. The differences were most striking and the results are summarised in Table II.

Table II. *24-hour cultures of pneumococcus in bicarbonate medium (M 6).*

Exp. no.	Type of organism	Growth in 24 hours	p_H	Gas liberation	Glucose disappeared %	Lactic acid formed %
P 31	S I	+	7·0	0	0·215	0·164
P 32	S II	0	7·2	0	0·065	—
P 33	R II	0	7·4	0	0·020	—
P 34	S III(*a*)	0	7·4	0	0·015	—
P 35	S III(*b*)	0	7·4	0	0·010	—
P 36	R III	+ + + + +	6·4	+ + +	0·992	0·779

Finkle [1931], working with washed pneumococcus suspensions, found that only the S I and R III forms effected aerobic glycolysis but whereas he found these two forms almost equal in activity the results above show that in growing cultures R III is much more active than S I.

The oxygen supply was restricted in the next series of experiments since Finkle found that all the types effected anaerobic glycolysis. With the bicarbonate media it is not possible to establish rigorously anaerobic conditions—the medium cannot be boiled to remove air since CO_2 is evolved and the medium becomes too alkaline. Cultures of the different types in bicarbonate medium were placed in a Bulloch's apparatus, and the air was replaced by passing hydrogen before incubation but the results were little different from those with aerobic cultures (Table III, col. 3). Phosphate medium (M 7) was therefore used, and in the anaerobic experiment the medium was heated in a boiling water-bath, cooled rapidly before inoculation and the culture incubated in a McIntosh and Fildes jar in the usual way.

Table III. *Glucose breakdown by different types of pneumococcus.*

	Bicarbonate medium M 6		Phosphate medium M 7	
Type	Aerobic %	Partly aerobic %	Aerobic %	Anaerobic %
S I	0·215	0·015	0·273	0·248
S II	0·065	0	0·065	0·245
R II	0·020	0	0·005	0·035
S III	0·015	0	0·158	0·225
R III	0·992	0·872	0·303	0·245

It will be seen in Table III that in anaerobic phosphate medium there is little difference in the amounts of glucose broken down by the different forms (except R II).

It should be remarked that in every case the amount of glucose breakdown has varied with the amount of growth of the organisms, as judged by the turbidity and deposit in the cultures.

Effect of inorganic phosphate.

The data in Table III suggested that inorganic phosphate might have an effect on glucose breakdown and Table IV gives the results of experiments conducted in two media both buffered with bicarbonate but one containing 0·4 % $Na_2HPO_4, 12H_2O$ as well.

Table IV. *Glucose breakdown in different media.*

Type	Bicarbonate medium M 6 (0·004 % inorganic P) %	Bicarbonate-phosphate medium M 8 (0·038 % inorganic P) %
S I	0·215	0·545
S II	0·065	0·930
R II	0·020	0·845
S III	0·015	0·425
R III	0·992	0·980

It will be seen that in every case there is more glucose breakdown in the media containing added inorganic phosphate except with R III where glucose breakdown was already maximal.

Experiments were next conducted with phosphate-free media (M 9 and M 10) from which the inorganic phosphates had been removed as described in a previous section. In cultures in media M 9 A and M 10 A the only inorganic phosphate present was derived from the culture used for inoculation. Varying amounts of inorganic phosphate were added to these basic media.

Table V. *Glucose breakdown in media containing varying amounts of inorganic phosphate.*

(Results expressed in g. per 100 cc.)

	Inorganic P content				
Type	A 0·0002 %	B 0·0006 %	C 0·0020 %	D 0·0072 %	E 0·0362 %
S I	0·095	0·129	0·240	0·269	0·260
S II	0·045	0·069	0·290	0·365	0·360
S III	0·065	0·189	0·175	0·240	0·272
R III	0·190	0·309	0·780	0·844	0·914
Mean	0·099	0·174	0·371	0·429	0·452

The amount of glucose broken down increases 3·7 times when the inorganic phosphorus content is increased from 0·0002 to 0·0020 %, and it increases 1·2 times when the inorganic phosphorus content is further increased from 0·0020

to 0·0362 %. As observed previously the amount of growth of the organisms as judged by the turbidity of the cultures appeared to be proportional to the sugar breakdown in each case. It should be emphasised that the media used in these experiments were adequately buffered with bicarbonate and the inorganic phosphate effect is not that of a buffer salt.

Discussion.

As with haemolytic streptococci so also with pneumococci glucose cleavage appears to be a relatively simple process, in contrast with the complexity of glucose metabolism in the case of many other bacteria. About 77·5 % of the glucose disappearing from cultures of either haemolytic streptococci or pneumococci may be recovered in the form of lactic acid, and this proportion seems not to vary over a considerable range of variation of cultural conditions. It is notable but not unexpected that glucose breakdown by pneumococci appears to be a similar process to that effected by haemolytic streptococci. This relatively simple method of glucose breakdown is probably accounted for in part by the absence of certain oxidising systems from the enzyme equipment of the organisms. Many other bacteria, for example, are able to oxidise lactic acid to carbon dioxide. But with the pneumococcus and haemolytic streptococcus the glucose molecule appears to break down to two molecules of lactic acid with a 78 % yield and the lactic acid formed appears not to be decomposed further in the cultures.

There are very marked and characteristic differences between the behaviours of the different types of pneumococcus. As would be expected from their different antigenic behaviour, chemical structure and virulence, their metabolic activities also differ. The yield of lactic acid from each molecule of glucose broken down in the cultures is the same with each type, but the actual amount of glucose broken down by the different types varies greatly. In aerobic cultures there was very much more glucose breakdown with R III pneumococci than with any other variant, S I came next in order of activity and the glycolytic activities of the other variants were much less. In anaerobic cultures the differences in glucose breakdown were much smaller. Finkle [1931] using washed suspensions of pneumococci in Ringer solution observed aerobic glycolysis with the R III and S I forms only, S I being only slightly less active than R III. Marked anaerobic glycolysis was observed with all the forms. In his experiments Finkle was presumably measuring the glucose activation of non-proliferating bacteria whereas in the present experiments glucose breakdown cannot proceed to any great extent unless bacterial growth occurs. In the present experiments the amount of glucose breakdown appeared to go *pari passu* with the amount of bacterial growth. In so far that the present results agree with those of Finkle it would seem that the growth of the pneumococci in the media used was dependent on glucose breakdown, but the discrepancy in the results, namely that the S I organisms were relatively much less active glycolytic agents in growing cultures, suggests that there may be a difference

in the growth requirements of the different forms apart from that of glucose breakdown. Whatever proves to be the explanation of the phenomenon the empirical fact remains that, in the glucose medium used, variants of both pneumococci and haemolytic streptococci may be differentiated by the amounts of glucose broken down in their cultures.

In the bicarbonate-phenol red-glucose medium used in certain experiments the difference in appearance of 24-hour broth cultures of the different types was very striking; the R III culture was milky in appearance with a heavy white deposit of bacteria, the phenol red was completely yellow and CO_2 was evolved with frothing, the other cultures were much less turbid, retained some of the pink colour of the indicator and showed no signs of effervescence or frothing.

The acidity produced is occasionally the limiting factor in the amount of glucose breakdown, and hence of bacterial growth, in pneumococcus cultures as with haemolytic streptococci. The superiority of bicarbonate over phosphate as a buffer in the cognate p_H range (say p_H 6·0 to 7·4) accounts for the increased glucose breakdown in the more actively glycolytic cultures when they are

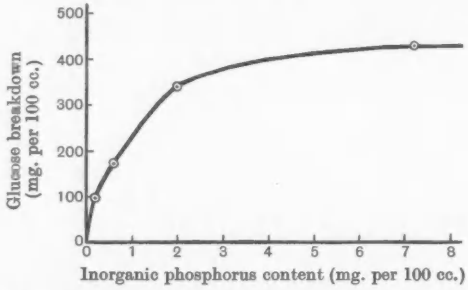

Fig. 1. Glucose breakdown in pneumococcus cultures containing varying amounts of inorganic phosphates.

buffered with bicarbonate rather than with phosphate. When, however, the inorganic phosphate concentration of the medium is too restricted the amounts of glucose breakdown and bacterial growth are very slight, but they are both stimulated when sodium phosphate is added. Thus in Fig. 1 are plotted the average figures obtained from Table V. By extrapolation it may be concluded that no growth of pneumococcus and no glucose breakdown would occur in this medium in the complete absence of inorganic phosphate. Friedlein [1928] found that the presence of inorganic phosphate was necessary for the growth of *B. coli* and *B. paratyphosus* B.

With regard to the mechanism of the effect of phosphate no data are yet presented but the analogies of the function of phosphates in glucose fermentation by yeast preparations [Harden and Young, 1906] and in the glucose metabolism of muscle suggest themselves. Virtanen [1924] sought for, but did not find, evidence of the formation of "zymophosphate" in the presence of living

Streptococcus lactis or of dried preparations, but in the case of dried preparations of *B. casei* ϵ, he noted a disappearance of inorganic phosphate during glycolysis and postulated the formation of "zymophosphate."

It should be noted that the beneficial effects of inorganic phosphate on the growth and glucose breakdown by pneumococci were observed in media sterilised by filtration. Deleterious effects may follow the addition of phosphate when the medium is afterwards autoclaved.

Summary.

1. Of the glucose disappearing from cultures of pneumococci about 78 % was recovered in the form of lactic acid.

2. The ratio of lactic acid formed to glucose broken down was unaffected by altering a variety of cultural conditions, and was the same as that observed in the case of haemolytic streptococci.

3. From the above it is concluded that glucose breakdown by pneumococci and haemolytic streptococci is a less complex process than with many other organisms.

4. There was more glucose breakdown in growing cultures with certain forms of pneumococcus than with others.

5. The presence of inorganic phosphate appears to play an essential part in bacterial growth and glucose breakdown, and not merely that of a buffer salt.

The author is indebted to Dr R. G. White and Dr E. W. Todd for their encouraging interest in the work.

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LVIII. NOTE ON AN ACCELERATING ACTION OF URIC ACID ON THE XANTHINE OXIDASE SYSTEM.

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(*Received February 29th, 1932.*)

In studying the effect of the xanthine oxidase system on other oxidising systems, it was desired to use some specific inhibitor of this oxidase in order to dissociate the effects produced by the oxidase from possible effects produced by other substances present in the enzyme solution. Dixon and Thurlow [1924] showed that uric acid retards the oxidation of hypoxanthine by xanthine oxidase in presence of methylene blue, and this acid therefore seemed to be a suitable inhibitor to use. On testing the effect of uric acid on the reduction of methylene blue by hypoxanthine and xanthine oxidase, however, it was found that the uric acid produced no inhibition but, instead, a marked acceleration of the reaction. At first, it appeared that some oxidisable impurity was present in the uric acid, but this possibility was excluded when it was found that other specimens of pure uric acid produced the same accelerating effect. At first sight, this result is directly contradictory to the results obtained by Dixon and Thurlow. On further consideration of the curves given in their paper, however, it appeared that an acceleration by uric acid of the activity of xanthine oxidase was not incompatible with the uric acid inhibitions which they described. They showed that with a constant amount of xanthine oxidase, on increasing the amount of hypoxanthine the rate of reduction of methylene blue remained constant until a certain critical concentration was reached, after which increasing the hypoxanthine concentration produced a marked and progressive inhibition in the rate of oxidation. They explained this inhibition as being due to the blocking of the enzyme surface by the excess of hypoxanthine, whereby the access of methylene blue to the surface is hindered and the reaction is retarded. By keeping the hypoxanthine concentration constant and adding increasing amounts of uric acid, Dixon and Thurlow found a similar increasing inhibition of the reaction, the inhibition in this case being ascribed to adsorption of uric acid on the enzyme surface preventing free access of methylene blue.

Assuming this explanation to be correct, it appears that the accelerating action of uric acid which I have observed can be explained in the following way. Let us consider a concentration of hypoxanthine sufficiently high to produce, with a given amount of enzyme, a considerable inhibition in its own oxidation.

On adding uric acid, part of the hypoxanthine adsorbed on the enzyme surface will be driven off and replaced by uric acid. That is, some of the one inhibitor, hypoxanthine, will be replaced by the other inhibitor, uric acid. If uric acid be a less powerful inhibitor of the reaction than hypoxanthine—and the inhibition-concentration curves of the two purines indicate that this is the case—then the result of adding uric acid should be the replacing of one inhibitor by a less powerful inhibitor, and a relative acceleration should be observed.

If this explanation be the true one, it would be expected that by keeping the enzyme constant and increasing the hypoxanthine to a concentration at which it produced an inhibition, the addition of uric acid would produce an inhibition which would be more than counteracted by its removing the more powerful inhibitor, hypoxanthine. That is, by increasing the concentration of hypoxanthine and keeping everything else constant, an inhibition by uric acid should be changed into an acceleration. That this actually occurs is shown by the following experiment.

Exp. 1. Thunberg vacuum tubes were used, and after filling, the tubes were evacuated, filled with nitrogen, re-evacuated and put in a bath at 37°. In all experiments described in this paper, 0·5 cc. of methylene blue (1 part in 5000 parts of water) was used in each tube, and the total volume of solution in the tubes was always 2·5 cc., this volume being made up where necessary by addition of the requisite amount of distilled water. The xanthine oxidase was the "whey preparation" described by Dixon and Kodama [1926], prepared by coagulating milk with rennin and half-saturating the whey with ammonium sulphate. 200 mg. of the dry preparation were dissolved in 5 cc. phosphate buffer and brought to p_H 7·4 with dilute NaOH. Both the hypoxanthine and the uric acid solutions were made by dissolving in slight excess of NaOH and bringing to p_H 7·4 with dilute HCl immediately before using. The hypoxanthine solution contained 1 mg. per cc. and the uric acid solution 2·5 mg. per cc. 0·2 cc. xanthine oxidase was used in each tube in this experiment.

Substances added	Time in mins. for M.B. reduction	Uric acid effect
1. 0·1 cc. hypoxanthine	7	
2. 0·1 cc. hypoxanthine + 1 cc. uric acid	14½	Inhibition
3. 0·4 cc. hypoxanthine	15½	
4. 0·4 cc. hypoxanthine + 1 cc. uric acid	11½	Acceleration
5. 0·8 cc. hypoxanthine	33½	
6. 0·8 cc. hypoxanthine + 1 cc. uric acid	14½	Acceleration

Comparing tubes 1 and 2 with tubes 3 and 4 or with 5 and 6, it is clear that, by increasing the hypoxanthine concentration 4 times or 8 times, the inhibitory effect of uric acid is changed into a marked acceleration. This experiment and the others described in this paper have been repeated several times with the same results.

Dixon and Thurlow showed that the critical concentration of hypoxanthine at which it commences to inhibit its own oxidation is dependent on the enzyme concentration: the more concentrated the enzyme solution, the

more hypoxanthine is necessary before the critical concentration is reached. By keeping the hypoxanthine concentration constant and decreasing the enzyme concentration, the hypoxanthine would become an inhibitor, and it might be expected therefore that under these conditions the inhibition by uric acid would again be changed into an acceleration. Exp. 2 shows that this actually occurs.

Exp. 2. The enzyme solution and the purine solutions were the same as those used in the previous experiment. 0·2 cc. of hypoxanthine was used in each tube. 0·3 cc. phosphate buffer was added to tubes 3 and 4 to keep the phosphate concentration the same in all the solutions.

Substances added	Time in mins. for reduction of M.B.	Uric acid effect
1. 0·4 cc. enzyme	$5\frac{3}{4}$	
2. 0·4 cc. enzyme + 1 cc. uric acid	$10\frac{1}{4}$	Inhibition
3. 0·1 cc. enzyme	46	
4. 0·1 cc. enzyme + 1 cc. uric acid	$24\frac{1}{2}$	Acceleration

It can be seen that, with the hypoxanthine kept constant, the reduction of the enzyme concentration to one-quarter has changed an inhibition by uric acid into a marked acceleration.

It is clear, therefore, that an inhibition by uric acid can be changed into an acceleration either by increasing the concentration of hypoxanthine or by decreasing the enzyme concentration. Whether an inhibition or acceleration is produced under given conditions by uric acid depends on the relationship between the concentrations of hypoxanthine and enzyme.

In Exp. 3 a similar effect was obtained, using fresh milk to supply xanthine oxidase.

Exp. 3. Each tube contained 0·25 cc. of fresh cow's milk. The hypoxanthine solution used in this experiment contained 10 mg. in 3 cc., the uric acid concentration being the same as in the previous experiments.

Substances added	Time in mins. for M.B. reduction	Uric acid effect
1. 0·03 cc. hypoxanthine	$5\frac{1}{2}$	
2. 0·03 cc. hypoxanthine + 1 cc. uric acid	$9\frac{1}{2}$	Inhibition
3. 0·6 cc. hypoxanthine	13	
4. 0·6 cc. hypoxanthine + 1 cc. uric acid	$10\frac{1}{2}$	Acceleration
5. None	∞	

It can be seen that, using milk, the inhibition and acceleration produced by uric acid are much smaller than those observed when purified xanthine oxidase is used. Using milk, it is necessary to increase the hypoxanthine concentration to a much higher figure in order to obtain an acceleration by uric acid. Both the hypoxanthine and uric acid appear to be adsorbed on the enzyme to a much smaller extent in milk, probably owing to the adsorption by the enzyme of other substances present in milk.

It would naturally be expected that, in the transition from an inhibition to an acceleration by uric acid, an intermediate stage would be reached at

which the addition of uric acid produced no effect on the reaction. This condition has been realised experimentally on more than one occasion.

Summary.

In the oxidation of hypoxanthine in presence of xanthine oxidase and methylene blue, the addition of uric acid may under suitable conditions produce a marked acceleration instead of an inhibition. The inhibiting action of uric acid on the reaction may be converted into an acceleration either by increasing the hypoxanthine or decreasing the enzyme concentration.

The accelerating effect appears to be due to the replacement on the enzyme surface of the inhibitor hypoxanthine by the less powerful inhibitor uric acid.

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LIX. THE DENATURATION OF PROTEINS.
VIII. THE EFFECT OF DENATURATION ON THE VISCOSITY OF THE SOLUTIONS OF CERTAIN PROTEINS.

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Although a considerable amount of work has been carried out on the measurement of the viscosities of various protein solutions, little attention has been paid to the determination of whether any change in viscosity occurs on denaturation.

Thus, it is only just possible to infer from the results of Pauli [1928] on various solutions, and those of Ettisch and Sachsse [1931] for egg-albumin, that an increase in viscosity occurs on denaturation. The present work was therefore undertaken to obtain more precise information, measurements being made separately on solutions of undenatured protein, and on solutions of deliberately denatured but unflocculated protein.

Measurements on undenatured solutions of egg-albumin have been made by Loeb [1924], who showed that the viscosity-p_H curve was a straight line parallel to the p_H axis, *i.e.* that the viscosity was independent of p_H. This has been confirmed in the present work, and furthermore it is shown that the same is true for solutions of oxyhaemoglobin.

Since the completion of the work described in the present paper an account has appeared of an investigation by Anson and Mirsky [1932] in which it is shown that denaturation is accompanied by an increase in viscosity. This is referred to later.

Experimental.

The proteins used were oxyhaemoglobin and crystalline egg-albumin, the former being prepared by a modification of the method of Schulz [1898], and the latter by the method of Sørensen [1917]. In both cases the proteins were freed from salt by prolonged dialysis[1]. The concentration of protein in the stock solutions was estimated by the method of Devoto as modified by Hopkins [1900].

[1] The dialysis was allowed to proceed for several months until two litres of the dialysate when boiled down to a volume of 2 cc. gave no precipitate with barium chloride solution.

The viscosities were measured by the times of outflow through the capillary tube of an Ostwald quartz viscosimeter. In all cases the "relative viscosity" given is the ratio of the time of outflow of a certain volume of protein solution to the time of outflow of the same volume of pure water through the same viscosimeter at the same temperature.

It was shown by Poiseuille that the flow of a liquid through a capillary is expressed accurately by the formula

$$V = \frac{KPr^4t}{l},$$

where V is the volume passing through the tube in time t, K is a constant characteristic of the liquid, P is the pressure, r is the radius of the tube, and l its length.

In terms of viscosity this equation becomes

$$V = \frac{\pi Pr^4t}{8l\eta},$$

where η is the viscosity of the liquid.

If therefore we wish to use relative viscosities, we must be satisfied that the liquids in question, *viz.* the protein solutions and water, follow Poiseuille's law, as otherwise the value obtained would not be independent of the instrument used. According to Grüneisen [1905] the simplest method for testing whether a liquid follows this law is by making observations of the times of flow at a given temperature when the pressure is varied. The product of pressure and time of flow will then be constant so long as the liquid follows Poiseuille's law.

The apparatus used for the test was that of Applebey as described by Hatschek [1928]. The liquids tested were water and a 3 % oxyhaemoglobin solution. In both cases a range of pressure was used which gave times of flow from 100 to 200 secs., *i.e.* a much greater range than that required for the subsequent readings. In both cases the product of pressure and time of flow was constant, as shown by Table I.

Table I.

Pressure (P) (cm. of water)	Time of flow (t) (secs.)	$P \times t \times 10^{-2}$
	Water	
15·77	102·3	16·1
13·27	121·7	16·2
12·27	131·6	16·1
10·57	153·0	16·2
10·07	160·2	16·1
9·07	184·2	16·7
	Mean value of $P \times t = 16{\cdot}2 \times 10^2$	
	Haemoglobin solution	
14·76	127·7	18·8
13·51	141·7	19·1
12·76	149·5	19·1
11·86	163·7	19·4
11·26	170·0	19·1
10·66	180·6	19·2
10·16	189·1	19·2
	Mean value of $P \times t = 19{\cdot}1 \times 10^2$	

Thus the flow of water and of protein solution up to a concentration of 3 % through the given viscosimeter follows Poiseuille's law. This means that the viscosities measured are true viscosities and may be regarded as having a definite significance from which deductions can be drawn with regard to the changes occurring during denaturation.

The viscosimeter was cleaned and dried by the method recommended by Barr [1931]. Constancy of temperature was obtained by using a thermostat at 25° regulated to ± 0·05°. The front of the thermostat was transparent, and the viscosimeter was clamped vertically in such a position that the liquid in it was always completely immersed below the level of the water in the thermostat, and also so that the marks on the instrument could be viewed directly through the window.

After the liquid had been introduced into the viscosimeter, the instrument was left in the thermostat for 10–15 minutes for the attainment of temperature equilibrium.

The times of flow were taken with a stop-watch correct to 0·1 sec. In all cases five readings were taken and a mean value obtained. For solutions containing 3 % protein the max. error amounted to ± 0·3 %; in the case of 0·2 % solutions the error did not exceed ± 0·2 %.

The method used for the p_H determinations was that of Harrison [1930] using the glass electrode. The temperature of the determinations was 18°. Harrison claims an accuracy of 0·02 of a p_H unit between the p_H values 1 and 12.

The viscosity of undenatured and denatured egg-albumin.

The volume of stock egg-albumin solution (prepared as described previously) required to give 15 cc. of a 3 % solution was measured out, and to it was added sufficient dilute sulphuric acid to make the volume up to 15 cc., the concentration of the acid being such as to bring the p_H of the solution to the required value. For measurements on undenatured egg-albumin, 10 cc. of this mixture were immediately pipetted into the viscosimeter which was then fixed in the thermostat, and after 10–15 minutes the mean time of flow was obtained as previously described.

In the case of the denatured egg-albumin, the 15 cc. of 3 % solution was heated on a water-bath to a temperature at which the process of denaturation was complete in about a minute at the p_H of the solution. This temperature was obtained by a study of the results of Lewis [1926] and Cubin [1929] and was in most cases between 80 and 90°. The solution was then cooled down rapidly and 10 cc. measured out for the viscosity determination as before. The mean of at least five readings was taken. The p_H of the solution was subsequently determined.

If the viscosity readings did not agree within the limits of experimental error, but showed a tendency to rise as time went on (owing to incipient flocculation), they were rejected. If no rise occurred, a sample of the solution was kept in the ice-chest for several days. If no visible flocculation occurred in this time it was assumed that the aggregation of molecules subsequent to denaturation had not occurred during the time taken for the viscosity measurements. If any flocculation became apparent in the solution even after 4 days, the readings were rejected.

As an added precaution, in case flocculation had occurred, but to an extent insufficient to be visible to the naked eye, one solution, after being kept in the ice-chest for 24 hours, was removed and its viscosity redetermined. The mean of six readings was 0·6 sec. higher than the previous mean, *i.e.* an increase of only 0·4 %. As the viscosity measurements are very sensitive to flocculation, this was conclusive evidence that flocculation had not even begun during the time taken for the determination of the viscosity. Thus we are justified in assuming that any change in viscosity can be attributed solely to the effect of denaturation.

Owing to the comparatively slow rate of denaturation at ordinary temperatures, it was possible to obtain the viscosities of undenatured solutions

from high p_H values down to p_H 2·0. Measurements for denatured solutions were made at $p_H < 2·9$, but between this p_H and the isoelectric point flocculation occurred and the results were accordingly rejected.

Viscosity of 3 % salt-free solutions of egg-albumin at 25°.

Mean time of flow for water = 133·3 secs. In all cases the mean time given below is the mean value of six readings.

Egg-albumin.

Undenatured solutions

p_H	Mean time of flow (secs.)	Relative viscosity
1·97	154·9	1·162
2·19	155·6	1·167
2·67	156·6	1·175
3·77	155·7	1·168
4·25	156·5	1·174
5·08	154·8	1·161
5·67	155·0	1·163
6·67	154·9	1·162
7·61	156·7	1·175
	Denatured solutions	
2·12	174·3	1·308
2·26	173·5	1·302
2·26	172·2	1·292
2·82	175·1	1·314
2·90	173·5	1·301

Inspection of the foregoing results indicates that the viscosity of solutions of undenatured crystalline egg-albumin is independent of p_H, the variations being due to experimental error only. This confirms the results of Loeb [1924]. Furthermore, the viscosity of denatured solutions of egg-albumin is similarly independent of p_H provided that incipient flocculation has not occurred.

The mean value of the viscosity of a 3 % solution of undenatured egg-albumin is 1·168, and of a 3 % denatured solution 1·303. The increase in viscosity which occurs on denaturation is constant and independent of p_H. In view of the constancy of viscosity over the p_H range 2·1 to 2·9, it is reasonable to conclude that even at the isoelectric point the viscosity of a 3 % solution of denatured egg-albumin is 1·303. This conclusion receives further support from the measurements on oxyhaemoglobin solutions which are given below.

Viscosity of solutions of undenatured and denatured solutions of oxyhaemoglobin.

These measurements were carried out in a manner similar to those already described for egg-albumin, the same precautions being taken to ensure that denaturation was not accompanied by flocculation even to the slightest extent. The work on 3 % solutions was performed by N. Booth[1] and the results which he obtained are given here for the sake of comparison.

[1] Unpublished work carried out in this laboratory.

Data of Booth for 3 % salt-free solutions of oxyhaemoglobin.

The mean time of flow for water in the instrument used by Booth was 130·0 secs.

Oxyhaemoglobin.

Undenatured solutions

p_H	Mean time of flow (secs.)	Relative viscosity
4·58	165·5	1·286
5·14	163·7	1·272
5·53	166·5	1·294
5·78	161·6	1·255
6·09	162·1	1·259
6·89	163·7	1·272
7·42	163·4	1·270
7·82	164·5	1·278
8·21	161·9	1·257
	Mean value of the relative viscosity =	1·272

Denatured solutions

2·38	201·2	1·564
3·83	201·6	1·567
4·50	211·6	(1·644)
5·07	204·1	1·586
	Mean value of the relative viscosity =	1·572

To confirm the results of Booth, and also to see whether the increase in viscosity on denaturation was dependent on the concentration of the solution, measurements were made on a series of more dilute solutions, *viz.* 0·2 % solutions. In this case we are dealing with quite small differences in viscosity, but that the results are as significant as those already obtained for the more concentrated solutions is seen when the following facts are taken into consideration.

The mean time of flow for 0·2 % undenatured solutions was about 2 secs. greater than that for water. The mean time for the denatured solutions was about 3·5 secs. greater than that for water. For these dilute solutions the separate readings agree far better than do those for the more concentrated solutions. This is shown by the fact that the highest and lowest single readings for the undenatured solution differed by only $\pm$ 0·5 sec., and for the denatured solutions by $\pm$ 0·4 sec. Also these various readings were made with separate solutions made up independently from the stock solution. Hence a difference of 1·5 secs. between the mean values for undenatured and denatured solutions respectively must be regarded as being a significant difference. This point is emphasised if the readings are plotted in the form of a graph, which is here omitted, however, for the sake of brevity.

In both cases therefore it will be seen that the viscosity of a given concentration of undenatured oxyhaemoglobin solution is constant and independent of p_H. The effect of denaturation on the solution is to cause a definite increase in viscosity, this increase being independent of the p_H of the solution. Consequently the effect of denaturation on viscosity is similar in the cases of oxyhaemoglobin and egg-albumin.

Viscosity data for 0·2 % salt-free oxyhaemoglobin solutions.

Temperature = 25°.
Mean time of flow for water = 133·3 secs.

Oxyhaemoglobin.

Undenatured solutions

p_H	Mean time of flow (secs.)	Relative viscosity
5·24	134·9	1·012
5·35	135·1	1·014
6·49	135·0	1·013
7·21	135·2	1·014
7·26	135·5	1·017
8·42	135·6	1·017
	Mean value of the relative viscosity = 1·0145	

Denatured solutions

2·29	136·8	1·026
2·41	137·1	1·028
2·68	136·6	1·025
2·71	136·9	1·027
2·86	136·6	1·025
3·02	136·7	1·025
3·46	136·7	1·025
4·16	136·7	1·025
	Mean value of the relative viscosity = 1·026	

Thus the experimental results confirm the conclusions just published by Anson and Mirsky [1932] that the viscosity of the protein solutions under consideration is increased by denaturation. It must be pointed out, however, that in the present work care has been taken to use denatured solutions in which the process of flocculation has not even commenced, whereas in general Anson and Mirsky must have been using solutions in which flocculation had proceeded at least to some extent. This is evidenced by the fact that some of their solutions were at a p_H quite close to the isoelectric point, and the present work has shown that in this case aggregation of the particles is inevitable.

At p_H 2–3 the relative viscosity of a 4 % denatured egg-albumin solution was found by Anson and Mirsky to be only of the order 1·5. In this range their work is most nearly comparable to our own as aggregation would not have occurred, so that increase in the viscosity due to the aggregation of the particles would not be experienced. As in our own case, with very acid solutions stiff gels were formed so that measurements of the increase in viscosity due to denaturation alone were impossible.

In view of the above considerations it seems that the present work must be regarded as demonstrating more concisely that the process of denaturation itself causes an increase in the viscosity of the protein solutions, as distinct from the subsequent further increase when aggregation of the particles in the denatured solution occurs.

Considering the three sets of viscosity measurements together, we see that in each case the viscosity of the denatured solution is constant over the whole range of p_H used. In two of the three cases the p_H ranges used for the undenatured and denatured solutions overlap, and even in the overlapping range the viscosities of the two solutions remain quite constant at their respective values. Thus, although it is not possible to measure the viscosity of a denatured but unflocculated protein solution at the isoelectric point, we are justified in concluding that the viscosity at this point will be the mean value which we have obtained for a considerable range of p_H. This conclusion will be employed later in the discussion of the significance of the viscosity results.

Dilatometer measurements.

A series of experiments was also undertaken using a dilatometer of the type described by Sreenivasaya and Sastri [1929] to determine whether the net volume of the protein solutions altered on denaturation. A mark was made near the lower end of the capillary tube and it was found that 30 cc. of liquid were required to fill the instrument to this mark.

The radius of the capillary was 0·125 cm. so that the cross-section was approximately 0·05 cm.^{2} Consequently an increase in the volume of the solution of 1 cc. would cause a rise in the capillary of 20 cm. Alternatively this means that a 1 % increase in the volume of the solution would cause a movement of the meniscus in the capillary tube of 6 cm. Consequently a small change of volume would cause a quite appreciable movement of the meniscus.

As the dilatometric measurements were to be made at 45° in order to obtain a suitable rate of denaturation, it was necessary to observe first of all what change in volume occurred when the instrument, initially filled to the mark at room temperature, was raised to a temperature of 45°. This was done by filling the instrument to the mark with distilled water at room temperature, and then placing it in the thermostat at 45°. The meniscus in the capillary tube rose for about 10 minutes, but when temperature equilibrium had been attained, the meniscus remained stationary. A mark was made on the capillary corresponding to this new position of the meniscus.

The dilatometer was emptied, dried thoroughly, and then filled to the lower mark on the capillary with a 3 % salt-free undenatured egg-albumin solution (p_H about 3·0) at room temperature, care being taken that no air bubbles were enclosed. The instrument was then clamped in the thermostat and the meniscus was observed through the window. In the first 10 minutes the meniscus rose to the upper mark on the capillary, but thereafter it remained stationary, although a control experiment showed that the protein was in process of being denatured.

This experiment was repeated at several different p_H values with the same result. There was the initial definite expansion due to the warming of the solution, but no subsequent volume change was noted during the time (one to several hours according to the p_H of the solution) required for the process of denaturation. A 3 % oxyhaemoglobin solution gave the same result.

From these experiments we conclude that no change occurs in the net volume of a solution of egg-albumin, or of oxyhaemoglobin on denaturation. The importance of this conclusion will be seen in the subsequent discussion.

Discussion.

As is well known Einstein [1906] was the first to attempt a mathematical treatment of the viscosity of a disperse system. Making several simplifying assumptions he arrived at the equation:

$$\eta = \eta_0 (1 + 2{\cdot}5\, \phi),$$

where η is the viscosity of the disperse system, η_0 that of the medium, and ϕ the aggregate volume of spheres in unit volume of the suspension. This formula, however, as Einstein himself points out, can only be used when ϕ is very small.

Various formulae have been suggested to overcome this limitation, in nearly all cases a value larger than 2·5 being given to the coefficient of ϕ, but none of these formulae allows specifically for any possible influence of the electric charge on the particles on the viscosity. It is obvious however that if we restrict ourselves to the isoelectric point for both the undenatured and the denatured material, the effects considered by Smoluchowski [1916] do not enter, and we are justified in employing the simpler type of formula.

Further than this, certain conclusions can be drawn from the viscosity data at the isoelectric point which do not demand a precise knowledge of the numerical coefficient of ϕ in the formulae of the Einstein type.

Thus at the isoelectric point we may employ the general equation,

$$\eta = \eta_0 (1 + k\phi),$$

where k is a constant.

Let us represent the viscosity of the undenatured solution at the isoelectric point by η_1, and of the denatured solution also at the isoelectric point by η_2.

Then $$\eta_1/\eta_0 = 1 + k\phi_{\text{undenatured}}$$

and $$\eta_2/\eta_0 = 1 + k\phi_{\text{denatured}}.$$

Whence by division we obtain

$$\frac{\phi_{\text{undenatured}}}{\phi_{\text{denatured}}} = \frac{\frac{\eta_1}{\eta_0} - 1}{\frac{\eta_2}{\eta_0} - 1}.$$

Thus the ratio $\frac{\phi_{\text{undenatured}}}{\phi_{\text{denatured}}}$ is independent of the value of k.

For 3 % egg-albumin solutions the value of $\frac{\phi_{\text{undenatured}}}{\phi_{\text{denatured}}}$ is $\frac{0{\cdot}168}{0{\cdot}303} = \frac{1}{1{\cdot}8}$.

For 3 % oxyhaemoglobin solutions it is $\frac{1}{2{\cdot}1}$ and for 0·2 % oxyhaemoglobin solutions it is $\frac{1}{1{\cdot}8}$.

These results indicate that on denaturation at the isoelectric point the volume of the dissolved protein unit increases, the increase being of the order 80–100 %.

The question now arises—do the protein units, in swelling, imbibe part of the continuous medium, or are they impermeable to it? In the latter case, the increase in volume would be transmitted to the total volume of the system, *i.e.* the process of denaturation would be accompanied by an increase in the net volume of the protein solution.

The increase in the value of ϕ on denaturing a 3 % egg-albumin solution is $\frac{0{\cdot}303 - 0{\cdot}168}{k}$ if we use the general equation. The maximum value given to k in any of the empirical formulae is 4·5, so that the increase in ϕ must be not less than

$\frac{0{\cdot}135}{4{\cdot}5}$ or 0·03. This means that if 1 cc. of a 3 % egg-albumin solution is denatured the volume of the protein units must increase by 0·03 cc. In the dilatometer experiments 30 cc. of the solution were used, so that the increase in volume to be expected is 0·9 cc. This would produce a movement of the meniscus of 18 cm. The dilatometric experiments however showed no appreciable expansion on denaturation. We may conclude therefore that the protein units are permeable to the continuous medium, *i.e.* as they expand on denaturation, the continuous medium flows in to such an extent that there is no change in the total volume of the solution.

Thus at the isoelectric point at least, the process of denaturation is accompanied by an increase in the particle size of the protein units, water flowing into the units during the swelling. Consequently we have an explanation of the observations of Chick and Martin [1910] that denaturation will not occur in the absence of water. This phenomenon in fact would be expected in view of the present work.

If we wish to obtain more precise knowledge of the absolute values of the size of the protein units before and after denaturation at the isoelectric point, it is necessary to employ the empirical equation proposed by Kunitz [1926] which is as follows:

$$\frac{\eta}{\eta_0} = \frac{1+0{\cdot}5\phi}{(1-\phi)^4},$$

which on expansion becomes

$$\eta/\eta_0 = 1 + 4{\cdot}5\,\phi + 12\phi^2 + 25\phi^3,$$

i.e. when ϕ is very small it tends to

$$\eta/\eta_0 = 1 + 4{\cdot}5\,\phi.$$

This corresponds to the Einstein formula with the coefficient of ϕ changed from 2·5 to 4·5; in other words it is the formula suggested by Hatschek [1910].

Kunitz has tested his formula and finds that it holds very well for as high concentrations as 50 % solutions of such substances as sugars, glycogen, caseinogen and rubber. Furthermore, for sugars and sulphur, the viscosity value agrees with the actual volume of substance in the dry state, whilst for caoutchouc in benzene ϕ agrees with the values obtained from the equation for osmotic pressure.

Applying Kunitz's formula in its simplified form to the results for 3 % undenatured egg-albumin we get

$$1{\cdot}167 = 1 + 4{\cdot}5\,\phi,$$

i.e. $\phi = 0{\cdot}037$.

If we assume the egg-albumin molecules to be spherical, an assumption supported by the ultra-centrifuge experiments of Nichols [1930], for a 3 % solution we obtain

$$\phi = \frac{3}{M} \cdot N \cdot \frac{4}{3}\pi r^3 \cdot \frac{1}{100},$$

where M is the molecular weight of egg-albumin, *viz.* 34,500, r is the radius and N is the Avogadro number, 6×10^{23}.

Equating these two expressions for ϕ we find that the radius of the undenatured albumin molecule (r) is $2{\cdot}57 \times 10^{-7}$ cm. as compared with Nichols's [1930] value of $2{\cdot}17 \times 10^{-7}$ cm. In a similar manner we find that the value of ϕ for 3 % denatured solutions of egg-albumin is 0·067, from which r is $3{\cdot}13 \times 10^{-7}$ cm.

Thus the viscosity data obtained can be interpreted to mean that on denaturation at the isoelectric point the dissolved units of albumin increase in size corresponding with an increase in the radius from 2·57 $m\mu$ to 3·13 $m\mu$.

The density of solid egg-albumin according to Arrhenius [1917] is 1·36. From this we can calculate a quantity ϕ' which would be the fraction of the volume of the solution occupied by the solute if the albumin underwent no change, *i.e.* did not become hydrated on being dissolved in water to give a 3 % solution.

Thus
$$\phi' = \frac{3}{1{\cdot}36} \cdot \frac{1}{100} = 0{\cdot}022.$$

But the viscosity data for 3 % undenatured solutions showed that ϕ is 0·037. This means that in a 3 % undenatured solution 0·022 cc. of protein is associated with 0·015 cc. of water, *i.e.* 1 cc. of protein is associated with 0·7 cc. water, or 1 g. of protein is associated with 0·5 g. of water. This value 0·5 is termed the "hydration factor" of the albumin. Incidentally this compares with the value 0·7 calculated by Arrhenius [1917] using the viscosity results of Chick [1914], and Chick and Lubrzynska [1914].

In the case of 3 % denatured egg-albumin solutions, by a similar calculation we find that 1·5 g. of water is associated with 1 g. of protein.

Passing now to the results for 0·2 % solutions of oxyhaemoglobin we find that for the undenatured protein at the isoelectric point, ϕ is 0·0033. If v is the volume of an individual dissolved protein particle, then

$$\phi = \frac{0{\cdot}2}{66800} \times 6 \times 10^{23} \times \frac{v}{100} = 0{\cdot}0033,$$

where 66800 is the molar or "micellar" weight of oxyhaemoglobin; from which v is found to be 184×10^{-21} cc.

The value of v given by Svedberg and Nichols [1927] is 110×10^{-21} cc. so that there is fair agreement between our value obtained from viscosity measurements and the value obtained from ultra-centrifuge measurements. On denaturation, the value of ϕ is 0·0058 from which the volume of an individual denatured particle at the isoelectric point is 323×10^{-21} cc.

Svedberg and Nichols [1927], taking the density of solid haemoglobin as 1·33, calculate that the volume of an individual particle should be 83×10^{-21} cc. Thus for undenatured particles 1 g. of protein is associated with 1·62 g. of water, and for denatured particles 1 g. of protein is associated with 3·85 g. of water.

From these figures we can infer that on denaturation at the isoelectric point oxyhaemoglobin undergoes a similar change to egg-albumin, *viz.* the protein particles distend by abstracting water from the continuous phase.

It is to be emphasised that the numerical values for the sizes of the undenatured and denatured protein units calculated above are intended to hold good only at the isoelectric point, since it is only at this p_H that we can be sure that there is no effect on the viscosity due to the electric charges on the protein particles.

Probably the most striking fact in connection with the viscosity is its independence of p_H, and the point is how this is to be interpreted in terms of the size of the colloidal unit. At first sight it appears that no electroviscous effect in the sense of Smoluchowski [1916] is operating, but at the present juncture it is not possible to state whether this is actually the case.

The general conclusion, however, that the denaturation of the proteins under consideration is accompanied by an increase in the size of the units, obviously holds at all values of p_H independently of the electroviscous effect.

Summary.

1. Viscosity measurements have been made on solutions of undenatured egg-albumin and oxyhaemoglobin, and on deliberately denatured but unflocculated solutions of the same proteins.

2. In both cases the viscosity of a given concentration of undenatured solution is constant and independent of p_H.

3. Similarly the viscosity of a given concentration of denatured solution is also constant and independent of p_H provided that no flocculation has occurred.

4. On denaturation, the viscosity of the solutions increases, the increase being the same at all values of p_H for any given concentration of protein.

5. Dilatometer measurements show that there is no change in the total volume of the protein solutions when these undergo denaturation.

6. It is shown that at the isoelectric point the process of denaturation is accompanied by an increase in the size of the protein unit, the volume of the denatured unit being approximately twice that of the undenatured. In the light of the dilatometer results it follows that the process of denaturation is accompanied by an increase in the amount of water associated with each individual protein particle, this conclusion being in accordance with the well-known fact that denaturation will not occur in the absence of water.

7. It is further shown that this conclusion is probably true at p_H values other than the isoelectric point, even allowing for the possible effect of the electric charge carried by the particles on the viscosity of the solution.

8. Briefly the process of denaturation from the physical standpoint consists in a change from the molecular to a micellar state (without aggregation however) on the part of each protein unit.

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LX. THE DETERMINATION OF VITAMIN D[1].

I. THE RELATIONSHIP BETWEEN GRADED DOSES OF A STANDARD SOLUTION OF VITAMIN D, ADMINISTERED TO YOUNG RATS ON A RACHITOGENIC DIET, AND THE ASH CONTENT OF THEIR BONES.

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Introductory.

The methods for the biological estimation of vitamin D, which are commonly used, are all based on a measurement of the calcification of the bones of rats, on a diet very low in phosphorus and high in calcium. The measurement of calcification is made either by X-ray photography of the epiphysis or growing end of a selected bone, or by staining the calcium deposited in it ("line" test), or by gravimetric analysis of the ash in certain selected whole bones. The first two methods measure essentially the same thing and the antirachitic treatment may be administered prophylactically or curatively; both these methods involve a certain subjective element in judging with the eye, by matching, the degree of calcification attained. The third method measures calcification in the whole bone, including the growing end; the test is usually prophylactic and there is no subjective element of error, but there might be an equal degree of error due to manipulation in the course of the analysis. The absence of any subjective element renders the third method attractive, though it is very much longer and more tedious than the other two.

For the application of any of these three methods some standard of comparison is desirable, in the form of a previously prepared scale, which shall relate a series of doses of a known standard preparation of vitamin D (cod-liver oil or irradiated ergosterol) to the degree of calcification produced and measured by any of the three methods mentioned. The preparation of such a scale, for use with the X-ray method, is described by Bourdillon *et al.* [1931] and by Everse and van Niekerk [1931], and for use with the "line" test by Bills *et al.* [1931] and by Dyer [1931]. No attempt has heretofore been made

[1] The three papers grouped under this heading describe the results of work undertaken at the instance of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council. The second paper includes, in addition, some account of a comparative study initiated by Dr Zilva who kindly placed his results at the disposal of the authors.

to construct a scale which shall correlate different values of percentage ash in the bone with different graded doses of vitamin D. Indeed Adams and McCollum [1928] seem to have reached the conclusion that it would not be possible to construct such a scale, for they conclude (p. 521) that "data obtained in the present investigation, however, indicate that there is scarcely a standard behavior of animals as evidenced by bone composition."

The construction of such a scale, correlating the percentage ash in the bones with graded doses of vitamin D, would have the added interest, if it could be done, that estimation of the percentage ash is a method which reveals the condition of calcification in the whole bone, while estimation by X-ray or "line" test reveals only what the eye can detect; from the former set of data, therefore, biological facts of fundamental interest may conceivably be deduced, while from comparable data obtained by means of the X-ray or "line" test, this is hardly likely to be possible. This point has, however, no bearing on the relative merits of the various methods as means for the evaluation of vitamin D.

The present paper describes an attempt to prepare such a scale and contains some other data which have a bearing on the general accuracy and application of the method, but it is not proposed to deal exhaustively with this aspect of the subject. Certain sources of error are inherent in the biological method, in whatever way the measurement of the degree of calcification may be made; these are, particularly, the variation of the stock of rats in sensitivity to rickets, the variation in the response of individual rats, even when litter-mates, to identical treatment, and variation in the rickets-producing potency of the basal diet or of certain elements in it. These sources of error have been discussed very fully by Bourdillon *et al.* [1931] who reached the conclusion that the variation in the responses of litter-mates to the same dose of vitamin D was the chief of all the sources of error. It might be expected that the responses of animals in a prophylactic experiment, in which the state of calcification of the whole bone is measured, would be less subject to individual variations, than would the responses of animals, already abnormal, in a curative experiment, where only calcification at the epiphysis is measured. Inspection of the results included in this paper shows, however, that the range of individual variation is also very high when the prophylactic method is used, and that it is not at all infrequent for a rat receiving a certain dose of vitamin D to show a lower calcification than another rat, its litter-mate, receiving half the dose.

The question, therefore, at once arises as to the number of rats which it is necessary to employ in testing each single dose, in order to give average results in which a series of doses will fall smoothly into line. Section I provides a small amount of data, which, although the experiments were not planned to answer that particular question, are relevant.

The writers have only been able to find in the literature one considerable series of ash estimations on the bones of rats, in which the values for the two hind legs were worked out separately [Jones and Robson, 1931]. With a view to throwing light upon the degree of error which is to be ascribed to the

manipulation of the worker, a large part of the results in this series has been worked out in that way; these results are dealt with in Section II.

Finally Section III deals with the actual construction of the curve correlating the ash content of the bones with the given doses of a standard solution of irradiated ergosterol.

The same series of rats described in Section III was also X-rayed at the end of the experiment, through the kindness of Dr R. B. Bourdillon and Miss H. M. Bruce, and the evaluation of this identical experimental series by the X-ray method is dealt with by them in the ensuing paper (p. 506).

Technique.

The rats used were of the Lister Institute piebald strain, aged 21–28 days, weighing 35–40 g. in the first experiment in Section I, and 40–45 g. in the other experiments.

Each rat was housed separately in a metal cage, with a fine wire grid as flooring. Cotton wool was given as bedding. Equal distribution of litters and, as far as possible of sexes, between the different experimental groups was strictly observed. The diet used was McCollum 3143 [McCollum *et al.*, 1921]. The rats received the experimental diet for a period of 28–33 days, the number of days being strictly the same for all rats in any one comparative series; they also received the given dose of vitamin D daily throughout the experiment, except on Sundays, the doses on Saturday and Monday being adjusted to make up the omission.

The standard used was one or other of the standard solutions of 0·01 % irradiated ergosterol, dissolved in inactive olive oil, prepared at the National Institute of Medical Research, Hampstead. In the two experiments in Section I, standards II and III were used; for the construction of the scale (Section III) only standard III was used, this being part of the solution now in use as the international standard. Both the original solutions and any dilutions were kept in the cold room at about 0° throughout, except when brought out for use. Dilutions were made by weight with olive oil as the diluent; dosage was so arranged that every animal in a given comparison received the same amount of olive oil. The oil was dropped into the animal's mouth from a capillary pipette, the drops given being the number calculated to give the required dose.

At the end of the experiment the rats were killed with coal gas, and after being X-rayed, the hind legs were removed and the bones (femur, tibia and fibula) were cleaned, broken across, dried, extracted with alcohol and ether, and ashed to constant weight. The results are expressed as the percentage ash in the dried extracted bone, but, for the average values on which the scale is based, they are also calculated as the percentage ash in the dry bone and as the A/R ratio, the latter being the value of the ash divided by the value of the organic part of the dried extracted bone (*i.e.* the difference between dried extracted bone and ash).

SECTION I. (By E. M. Hume and M. Pickersgill.)

The original object of the two experiments described in this section was to compare the antirachitic values of two different specimens, Nos. II and III, of standard solution of irradiated ergosterol. From this particular point of view the results of the experiments are not relevant to the subject of this paper and it may be taken from evidence published by Bourdillon, Bruce and Webster in the third paper in this series (p. 522) that the two samples, II and III, were of like antirachitic value. The results do, however, serve as a preliminary observation, determining roughly the number of animals which it is desirable to use for any single dose in a comparison, in order to obtain satisfactory average values correlating dose and degree of calcification. They also yield information as to the size of dose which it would be desirable to use in such prophylactic experiments.

Exp. 1. Daily doses of 0·05, 0·1 and 0·2 international units of vitamin D, being the equivalent of 0·005, 0·01 and 0·02 γ (1 unit = 0·1 γ) of irradiated ergosterol, of each of the solutions II and III, were chosen for administration to groups of rats. There was also a group of negative controls receiving olive oil only and a group of positive controls receiving 2·0 units (solution III). Each of these 8 groups contained only 4 animals; 4 litters of 8 animals each were used so that each group contained one animal from each of the 4 litters.

Table I. *Percentage ash in the bones of rats, on a rachitogenic diet (McCollum 3143) and receiving various doses of two solutions of irradiated ergosterol (solutions II and III) of approximately equal strength, prophylactically for 30–35 days.*

Results calculated as percentage ash in dried fat-extracted bone.

			Daily dose of irradiated ergosterol expressed in units of vitamin D						
			0·05		0·1		0·2		2·0
Litter	Sex	0	Solution II	Solution III	Solution II	Solution III	Solution II	Solution III	Solution II
1539	♂	25·5	—	—	46·0	35·5	38·5	39·3	45·5
	♀	—	40·1	40·0	—	—	—	—	—
1542	♂	24·9	33·5	31·8	—	—	46·7	40·0	44·5
	♀	—	—	—	38·6	35·9	—	—	—
1537	♂	27·8	30·1	28·7	32·7	31·5	—	—	—
	♀	—	—	—	—	—	32·5	37·4	42·3
1557	♀	Lost	31·5	39·8	40·2	34·8	41·0	44·1	52·5
	Average	26·1	33·8	35·1	39·3	34·4	39·7	40·3	46·2
Combined av., solutions II and III		26·1	34·4		36·8		40·0		46·2

The results, expressed as percentage ash in the dried extracted bone (see Table I), are typical of what might be expected; there is great variation between individual values for the percentage ash in rats receiving the same dose, and great irregularity within the same litter, so that higher doses quite frequently give lower values than lower doses. On averaging the values from the 4 animals in each group, the results showing the degree of calcification corresponding with the different doses begin to fall into series, although the value for the dose

of 0·1 unit (solution II) is almost exactly the same as the value for twice that dose, and the value for the dose of 0·1 unit (solution III) is less than the value for half that dose. If, however, solutions II and III are regarded as being of the same antirachitic strength (as there is every reason to believe that they were), then results derived from 8 animals on each dose are available for calculating the average value for the percentage ash corresponding with each of the doses of 0·05, 0·1 and 0·2 units of vitamin D. When the results are averaged in this way, a much smoother series is obtained in which the values are at least nowhere reversed.

Exp. 1 therefore suggests that the number of animals in an experimental group should be more than 4 and probably at least 8.

Exp. 2. In the second experiment, the original object was again to compare the antirachitic values of the two standard solutions II and III, by comparing the results obtained from groups of 10 animals on a single dose. At the same time it was desired to determine the variation in the average result, which might be given by two groups of 10 animals receiving the same dose of the same solution. One group of 10 animals therefore received a certain dose of solution II, while two groups of 10 animals, comparable with the first in litter and sex, received the same dose of solution III. In effect, therefore, taking the two solutions II and III as being of equal antirachitic potency, the experiment should show the variation in the average result which is obtained when three comparable groups of 10 comparable animals are comparably treated.

The dose selected was one of 0·1 international unit of vitamin D daily, a dose which in the previous experiment had resulted in a suitably intermediate degree of calcification.

Reference to Table II shows that a generally higher level of calcification was attained in this experiment than in the previous one with the same dose,

Table II. *Percentage ash of the bones of rats on a rachitogenic diet (McCollum 3143) and receiving a daily dose of 0·01 γ (= 0·1 unit) of irradiated ergosterol, as two solutions II and III, of approximately equal strength, prophylactically for 30–35 days. Treatment of groups A and B, receiving solution III, was identical.*

No. of litter	Sex	Percentage ash in the dried, fat-extracted bone		
		Solution II	Solution III group A	Solution III group B
1679	♂	40·0	43·1	42 2
	♀	40·2	43·8	42·7
1682	♂	51·6	41·7	39·8
	♂	42·4	36·8	42·1
	♀	38·1	41·8	36·7
1680	♂	44·5	52·4	47·6
	♂	—	—	41·7
	♀	45·1	45·8	—
	♀	49·6	50·6	51·1
1691	♂	41·3	36·7	41·3
	♀	37·7	38·8	38·0
	Average	43·05	43·15	42·32

but the percentage ash in the dry extracted bone in this series is still much below the maximum attainable on the rachitogenic diet, when optimal antirachitic treatment is given. Though the individual values vary very widely, particularly within one litter (No. 1682), yet the average values (43·05, 43·15 and 42·32 %, in the three similar series) are very close together. It may therefore be concluded that where groups of as many as 10 comparable animals are used for each dose of material tested, an average result will be obtained upon which reliance may be placed and that with a graded series of doses, average results will be obtained which will arrange themselves in a fairly smooth series. A similar conclusion was reached by Sherman and Stiebling [1929]. They used groups of 10 or more rats and determined the content of calcium in the femur and concluded that reliance could be placed on the average result yielded by such groups, but the diet and conditions of their experiment were so different from the ones just described that argument from such data to the present case would not be valid.

The difference between the value obtained for the percentage ash content of the bones corresponding with a dose of 0·1 unit of vitamin D daily, in Exp. 1 (Table I, ash in dried fat-extracted bone, average of 8 rats, 36·8 %) and in Exp. 2 (Table II, ash in dried fat-extracted bone, average of 30 rats, 42·8 %) has already been pointed out, and this difference offers another example of the variation in results which is liable to be obtained in this kind of work when carried out at different times. Whether such a variation in the response of the experimental animals at different times is due to variation in the animals' reserves of vitamin D, or to variation in the rickets-producing power of any of the constituents of the basal diet or to some other unrecognised cause, is at present not known, but it is one of the greatest causes of difficulty in the biological estimation of vitamin D.

SECTION II. (By E. M. Hume and M. M. Gaffikin.)

Any evidence as to the measure of accuracy which may be expected in the actual manipulation of preparing and ashing the bones, in the method under discussion, is desirable. Estimations of ash content in the bones of the two hind legs of each rat were, therefore, carried out separately, in a large part of the series. The values for about 90 animals are available and are set out in Table III.

While the present work was in progress, a similar set of 50 estimations, made separately on the two hind legs of the same rat, was published by Jones and Robson [1931]. Their results show a variation very comparable with that obtained in the present series. The average difference between estimations of percentage ash in the dried extracted bone, carried out separately on pairs of legs, is in their case 0·56 % and in the present series 0·50 %.

Relatively to the error due to the variation in the individual responses of the rats, this degree of error is small. For instance, if for the 20 pairs of rats receiving doses of 0·1 and 0·2 unit of vitamin D (see Table IV A) the average

Table III. *Comparison between the percentage ash in the dried, fat-extracted bones (femur and tibia) of the right and left legs of rats, estimated separately.*

No. of rat	Percentage ash in dry, extracted bone: L. Tibia and femur	R. Tibia and femur	No. of rat	Percentage ash in dry, extracted bone: L. Tibia and femur	R. Tibia and femur	No. of rat	Percentage ash in dry, extracted bone: L. Tibia and femur	R. Tibia and femur	No. of rat	Percentage ash in dry, extracted bone: L. Tibia and femur	R. Tibia and femur
834	39·4	39·7	856	49·9	45·7	879	41·2	42·1	902	43·9	44·8
835	38·4	38·8	857	41·6	41·6	880	41·3	40·3	903	44·0	43·0
836	41·9	40·4	858	41·1	41·7	881	39·4	38·6	904	43·9	44·0
837	40·3	40·2	859	44·7	43·9	882	47·0	48·0	905	47·8	47·8
838	47·6	47·8	860	45·4	45·2	883	47·6	47·3	906	37·8	38·2
839	43·6	43·2	861	48·2	48·8	884	50·8	51·7	907	49·4	49·0
840	47·3	47·6	862	39·6	40·5	885	49·5	49·2	908	48·7	49·1
841	42·5	42·8	863	48·3	47·5	886	48·6	48·7	909	44·7	44·2
842	47·2	46·4	864	42·5	43·5	887	48·0	48·3	910	40·2	39·6
843	47·7	48·2	865	39·9	40·1	888	44·8	45·3	911	43·9	43·8
844	44·8	44·1	866	42·8	44·0	889	42·8	42·1	912	44·4	44·0
845	43·1	43·5	867	47·8	47·9	890	45·2	44·5	913	46·6	46·2
846	37·9	37·9	868	47·3	47·6	892	41·4	41·7	914	42·9	43·6
847	41·2	40·5	869	46·3	47·2	893	46·9	47·2	925	45·3	45·8
848	43·8	44·5	870	41·1	40·4	894	41·5	41·3	926	32·6	32·5
849	42·5	42·6	872	48·2	48·3	895	43·5	43·0	927	43·0	42·9
850	48·7	48·8	873	47·9	48·1	896	46·9	47·9	928	43·1	43·7
851	47·8	46·9	874	46·9	47·7	897	35·1	34·8	929	27·0	27·9
852	48·6	48·9	875	50·6	49·7	898	50·0	49·6	930	34·7	34·3
853	43·4	43·5	876	52·2	51·9	899	47·5	48·7	931	39·3	38·5
854	39·6	40·1	877	52·3	52·5	900	38·9	39·3	932	38·9	39·2
855	49·3	50·2	878	53·7	53·4	901	46·9	46·6	933	44·8	44·7

difference from the mean for the differences between pairs be calculated, it is found to be as large as 1·5 % of ash value, while the similar value for the doses of 0·1 and 0·05 unit is 2·6 % of ash value.

The question of errors is further considered by Bourdillon and Bruce in the ensuing paper (p. 506).

Section III. (By E. M. Hume and M. M. Gaffikin.)

The preparation of the scale.

In preparing a scale which should best show the relation of the calcification of the bones of rats on a rachitogenic diet to a series of graded doses of vitamin D, the selection of the doses is of paramount importance.

Dutcher *et al.* [1925] constructed charts, reproduced in Fig. 1 *a*, showing the progressive changes taking place with age in the calcification of the bones of young rats, maintained for periods varying from 0 to 8 weeks on a rachitogenic diet of the low phosphorus type (Steenbock 2965), both uncorrected and corrected with an optimal supply of vitamin D. They compared these with a similar chart, showing the corresponding values for young rats after similar periods of time on a normal diet. The values for the ash content of the bones thus obtained for rats on the corrected rachitogenic diet may be taken as the maximum which could be attained, the insufficiency of the phosphorus in the diet acting as a limiting factor which made the attainment of normal calcification impossible. Although the basal diet used by Dutcher *et al.* was not the same as that used by the writers, it was of the same type, and the young rats were similar in size and age, so that the values of Dutcher *et al.* make a very useful approximate

standard of reference. It is true that their curves are constructed using only a small number of animals, and the curve for rats on the uncorrected rachitogenic diet could certainly have no universal application, yet the values for rats on the normal diet and for rats on the corrected rachitogenic diet would be less likely to be greatly influenced by variations in the sensitivity of the rats to rickets.

Table IV A. *Percentage ash in the dried fat-extracted bones of young rats on 'McCollum's rachitogenic diet and receiving various doses of irradiated ergosterol, prophylactically for 24–33 days.*

No. of litter	Sex	Dose of irradiated ergosterol in international units of vitamin D (1 unit = 0·1 γ of irradiated ergosterol) 0	0·01	0·02	0·03	0·05	0·08	0·1	0·2	0·32	0·64	1·28
1826	♀	41·1	—	40·7	41·6	—	41·1	—	45·2	—	—	—
	♀	—	38·3	—	—	42·2	—	41·3	—	44·1	—	—
1827	♀	—	—	—	—	38·7	—	39·3	—	41·4	—	—
	♀	36·2	34·3	42·2	38·9	—	39·7	—	44·1	—	—	—
1914	♀	39·5	38·6	—	41·2	40·3	—	42·7	43·4	—	—	46·8
	♀	—	—	—	—	—	—	—	—	47·5	47·7	—
1917	♀	—	—	—	—	—	—	44·5	42·6	—	—	—
	♀	43·3	37·9	—	40·9	44·2	—	—	—	48·8	48·0	47·4
1915	♀	39·9	41·6	—	41·4	—	—	—	—	44·3	—	—
	♀	—	—	—	—	43·5	—	45·3	45·3	—	48·8	49·8
1943	♀	—	—	—	—	43·0	—	—	—	46·8	48·5	47·9
	♀	40·1	47·9	—	40·0	—	—	43·4	47·5	—	—	—
1954	♀	40·8	45·3	—	48·0	—	—	52·1	52·4	—	48·3	50·2
	♀	—	—	—	—	47·3	—	—	—	53·6	—	—
1963	♀	41·7	40·8	—	39·0	47·5	—	47·5	49·4	48·7	—	—
	♀	—	—	—	—	—	—	—	—	—	51·3	48·2
1965	♀	—	—	—	42·5	—	—	—	43·3	—	45·1	47·1
	♀	44·9	43·8	—	—	41·6	—	41·4	—	47·4	—	—
1991	♀	38·0	39·9	—	43·9	44·2	—	43·3	—	—	—	—
	♀	—	—	—	—	—	—	—	44·5	46·4	49·2	48·9
1828	♀	32·6	31·8	—	33·5	—	40·7	38·3	40·3	43·1	—	—
	♀	—	—	38·1	—	39·7	—	—	—	—	—	—
1834	♀	34·6	38·1	36·8	36·7	39·3	39·6	—	—	—	—	—
	♀	—	—	—	—	—	—	38·7	40·4	41·2	—	—
1854	♀	—	—	—	—	36·7	—	—	—	—	—	—
	♀	32·5	33·7	36·0	36·5	—	39·5	45·0	46·0	45·8	—	—
1869	♀	—	33·8	31·9	31·4	—	—	—	—	—	—	—
	♀	33·7	—	—	—	31·4	36·4	34·6	41·4	43·3	—	—
1867	♀	—	—	—	—	—	—	—	31·7	37·3	—	—
	♀	28·4	33·1	28·5	27·0	27·1	30·3	34·9	—	—	—	—
1875	♀	—	—	36·4	—	38·2	—	44·4	—	41·6	—	—
	♀	31·0	37·3	—	38·8	—	39·6	—	47·4	—	—	—
1988	♀	35·0	39·1	—	—	44·4	—	43·5	44·0	47·8	—	—
	♀	—	—	—	48·1	—	—	—	—	—	49·8	46·8
2057	♀	—	—	—	—	—	—	—	—	43·4	45·6	43·0
	♀	27·5	32·6	—	34·5	38·9	—	39·1	44·8	—	—	—
2060	♀	31·3	30·1	—	36·1	39·7	—	43·2	45·4	46·3	—	—
	♀	—	—	—	—	—	—	—	—	—	48·8	48·2
2095	♀	24·9	29·1	—	32·6	35·1	—	—	41·9	44·1	—	—
	♀	—	—	—	—	—	—	40·1	—	—	47·2	47·6
Av. of 20 litters ...		35·9	37·4	—	38·6	40·2	—	42·2	44·1	45·2	—	—
Av. of first 10 litters; 0 dose, ash over 35 %		40·6	40·8	—	41·7	43·3	—	44·1	45·8	46·9	—	—
Av. of last 10 litters; 0 dose, ash under 35 %		31·2	33·9	—	35·5	37·1	—	40·2	42·3	43·4	—	—
Av. of 8 litters including 0·02 and 0·08 unit doses		33·8	35·1	36·3	35·6	36·7	38·4	39·6	42·1	42·2	—	—
Av. of 12 litters including 0·64 and 1·28 unit doses		37·3	38·9	—	40·7	42·5	—	43·9	45·4	47·1	48·2	47·7

Table IVB. *The same series of averages, calculated as the A/R ratio.*

	Dose of irradiated ergosterol in international units of vitamin D (1 unit = 0·1 γ of irradiated ergosterol)										
	0	0·01	0·02	0·03	0·05	0·08	0·1	0·2	0·32	0·64	1·28
Av. of 20 litters ...	0·58	0·61	—	0·64	0·69	—	0·74	0·80	0·83	—	—
Av. of first 10 litters; 0 dose, ash over 35 %	0·69	0·70	—	0·72	0·77	—	0·80	—	0·89	—	—
Av. of last 10 litters; 0 dose, ash under 35 %	0·46	0·52	—	0·56	0·60	—	0·68	0·74	0·77	—	—
Av. of 8 litters including 0·02 and 0·08 unit doses	0·52	0·55	0·58	0·56	0·59	0·63	0·66	0·74	0·73	—	—
Av. of 12 litters including 0·64 and 1·28 unit doses	0·61	0·65	—	0·70	0·75	—	0·79	0·84	0·90	0·93	0·91

Table IVC. *The same series of averages, calculated as percentage ash in the dry non-extracted bone.*

Av. of 20 litters ...	31·7	32·6	—	34·0	35·1	—	37·0	37·8	38·4	—	—
Av. of first 10 litters; 0 dose, ash over 35 %	34·9	35·8	—	36·9	37·9	—	39·0	39·7	40·6	—	—
Av. of last 10 litters; 0 dose, ash under 35 %	28·4	29·4	—	31·1	32·3	—	34·9	35·9	36·1	—	—
Av. of 8 litters including 0·02 and 0·08 unit doses	29·4	29·9	32·6	31·3	32·2	33·8	34·6	35·9	36·3	—	—
Av. of 12 litters including 0·64 and 1·28 unit doses	33·1	34·4	—	35·8	37·1	—	38·5	39·1	39·7	40·8	39·3

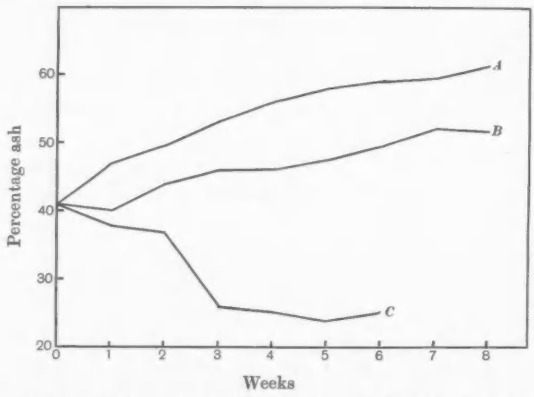

Fig. 1 *a*. (Reproduced by kind permission from the paper of Dutcher, Creighton and Rothrock [1925].) "Bone ash expressed in per cent. of dry extracted femurs." Values obtained by killing groups of about 4 rats at weekly intervals: curve *A*, rats on normal diet; curve *B*, rats on Steenbock's (2965) rachitogenic diet, irradiated; curve *C*, rats on Steenbock's (2965) rachitogenic diet, without addition of vitamin D in any form.

Consideration of Dutcher's values and of those set out in Table I suggests that a series of doses ranging from one rather larger than 0·2 unit down to no dose at all should cover the range of calcification possible of attainment on the rachitogenic diet.

For the present purpose the number of doses between which comparison

could fairly be made was limited by the number of rats which could regularly be obtained in one litter, since it was desired to make the groups for each dose absolutely uniform in their litter constitution. The maximum number of young rats which could regularly be obtained in one litter was found to be 9, and it was therefore decided to select 9 doses, one animal from each litter receiving each dose.

The sexes were distributed as regularly as possible amongst the groups. Reference to Table IVA shows that, in the full series of 20 litters examined, the animals on each of those doses for which 20 animals were used include either 11 males and 9 females, or 10 of each sex.

As it appeared from the values obtained in Exp. 1 that the response to increase of dose was much greater with smaller doses than with larger doses, the smaller doses were spaced more closely than the larger ones. The doses chosen were therefore 0, 0·01, 0·02, 0·03, 0·05, 0·08, 0·1, 0·2 and 0·32 international units of vitamin D.

When 8 litters, *i.e.* 8 animals on each of the 9 doses had been used, the average results were plotted, and it was found that the smaller doses appeared to be somewhat too close together and that one or two larger doses might give useful information. Doses of 0·02 and 0·08 units were therefore omitted in the later experiments and doses of 0·64 and 1·28 units were substituted. In all 20 litters were used, the 7 doses of 0, 0·01, 0·03, 0·05, 0·1, 0·2 and 0·32 units being represented in all these litters, so that for each of these doses the average value for the percentage ash derived from the bones of 20 animals is available for the construction of the curve. For the doses of 0·02 and 0·08 units, the average of only 8 animals is available, and for the doses of 0·64 and 1·28 units, the average from 12 estimations.

The values thus obtained, calculated as percentage ash in the dried, fat-extracted bone, are set out in Table IVA. The corresponding average values, expressed as percentage ash in the dry bone and as A/R ratio, have also been calculated and are included in Tables IVB and C.

In Fig. 1 the curves are plotted showing the average calcification values for the 7 doses for which 20 animals were used, the values for calcification being calculated as percentage ash in the dried, fat-extracted bone (curve a), as percentage ash in the dry bone (curve a_1) and as the A/R ratio (curve a_2). In the further analysis of the results only the values expressed as percentage ash in the dried, fat-extracted bone are used, but by reference to Table IVB and C values calculated in either of the other two ways can be substituted. Comparison of the 3 curves in Fig. 1 shows that those derived from the values for the dried fat-extracted bone and from the A/R ratio are, as would be expected, practically identical in form. The curve plotted with the values derived from the dry bone is somewhat different. Inspection of the amount of fatty material extracted from the bone by ether and alcohol shows that this amount is larger for animals receiving higher doses of vitamin D than for those receiving lower doses. For the doses of 0–0·1 unit, the percentage fat extracted from the dry bone

lies between 12 and 13 but for doses of 0·2 and 0·32 units it rises to 14·4 and 15·7 respectively, thus tending to depress the value for the percentage ash in the dry non-extracted bone for these higher doses.

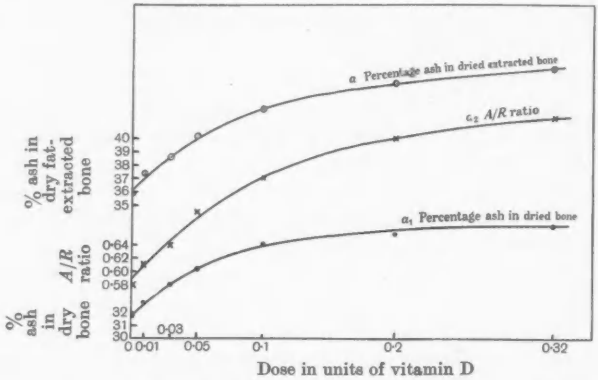

Fig. 1. Average values for the calcification of the bones of groups of 20 rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0–0·32 international units) of the international standard solution of vitamin D (1 unit = 0·1 γ irradiated ergosterol). Calcification values calculated as: curve a, percentage ash in the dried extracted bone (Table IV A, first line of averages); curve a_1, percentage ash in dried bone (Table IV C, first line of averages); curve a_2, A/R ratio (Table IV B, first line of averages).

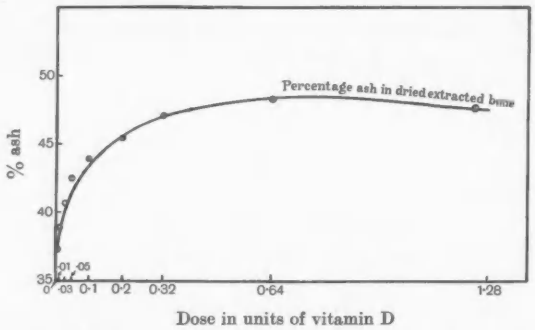

Fig. 2. Average values for the percentage ash in the dried extracted bones of groups of 12 rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0–1·28 international units) of the international standard solution of vitamin D (1 unit = 0·1 γ irradiated ergosterol) (Table IV A, fifth line of averages).

In Fig. 2 the curve is plotted for the series of values derived from 12 animals for each dose, and this includes the two higher doses of 0·64 and 1·28 units (see Table IV A, last line of averages). The percentage ash for these 2 doses is 48·2 and 47·7 % respectively, while that for the dose of 0·32 unit, in the same series, is 47·1 %, showing that the maximum calcification attainable has already been reached in this series with the dose of 0·64 unit.

The form of the curve thus plotted in Fig. 2 suggests a logarithmic relationship between the two variables.

The question at once arises, can this curve be accepted as a standard, relating the responses of all young rats of a given age and weight, on the given diet, to a given series of doses of vitamin D? And if not, how are similar curves, constructed in other laboratories, with rats bred under other conditions, likely to differ from it? The greatest difference will certainly be due to the varying resistance which different stocks of rats, or the same stock of rats at different times, will offer to the decalcifying action of the rachitogenic diet. The great variation which exists in sensitivity to rickets in an apparently uniform population of young rats is shown by the wide variation in the levels of calcification attained by the rats receiving no dose in the present series (see Table IV A); this level ranges from 24·9 to 44·9 % ash content of the bones.

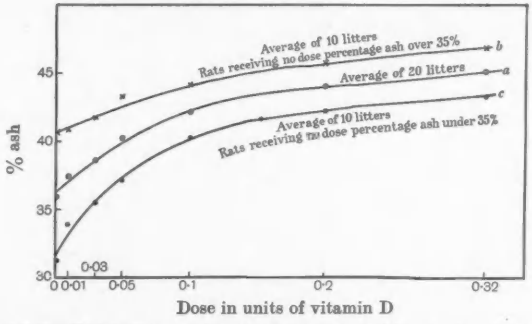

Fig. 3. Average values for percentage ash in the dried extracted bones of groups of young rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0–0·32 international units) of the international standard solution of vitamin D (1 unit = 0·1γ irradiated ergosterol): curve *a*, average values from 20 litters, *i.e.* 20 animals for each dose (same curve as Fig. 1, curve *a*) (Table IV A, first line of averages); curve *b*, average values from 10 litters, *i.e.* 10 animals for each dose, being that half of the series used for curve *a* in which the animals receiving no dose showed a percentage ash over 35 % (Table IV A, second line of averages); curve *c*, average values from 10 litters, *i.e.* 10 animals for each dose, being that half of the series used for curve *a* in which the animals receiving no dose showed a percentage ash under 35 % (Table IV A, third line of averages).

The present series of 20 litters can, by selection, be divided into two series, of which one, comprising the first 10 litters in Table IV A, shows a percentage ash of over 35 % for the rats receiving no dose of vitamin D, while the other series, comprising the last 10 litters in Table IV A, shows a percentage ash of under 35 % for the rats receiving no dose. Inspection of the serial numbers of these 20 litters shows that the more and less rachitic litters tend to occur in batches, but their occurrence at any given time has not as yet been correlated with any known set of environmental conditions.

In Fig. 3 the average values obtained with these two series of 10 litters are plotted against the doses, as two separate curves, together with the curve obtained with the whole 20 litters, which is reproduced from Fig. 1 for the sake of comparison. The 3 curves thus produced do not run parallel with one

another at different levels of calcification. For the higher doses they appear to do so, but for the lower doses the calcification of the more rachitic series of rats is relatively much lower than that of the less rachitic series.

Curves published by Dyer [1931], correlating doses of vitamin D with degrees of healing, as judged by the "line" test, show a similar divergence when a division is made into a more and a less rachitic series, although measurement in that case is made of deposition of calcium at the epiphysis of the bone, and in the present instance of prevention of decalcification in the whole bone.

Interpretation of the scale.

If the average values derived from estimations on 12 animals for the percentage ash in the dried fat-extracted bone of rats receiving doses of 0·03 to 1·28 units of vitamin D (Table IVA, fifth line of averages; see also Fig. 2) are plotted against the logarithms of the doses (Fig. 4, curve (*e*)), the points are

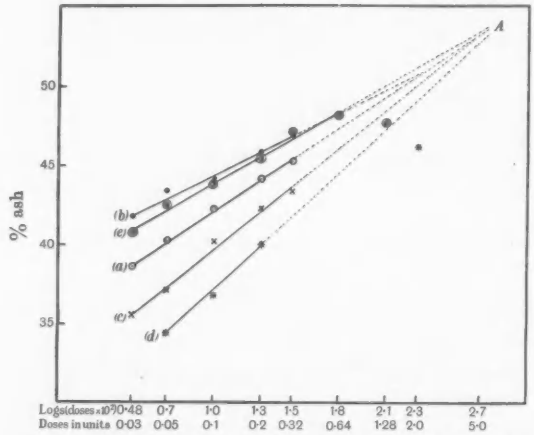

Fig. 4. The ash percentages, plotted against the doses in Figs. 2 and 3, are replotted against the logarithms of the doses. Curves (*a*), (*b*) and (*c*) correspond with curves *a*, *b* and *c* in Fig. 3. Curve (*d*) is plotted from the series of average values for 8 animals on each dose (4 only for the dose of 2 units), taken from Table I. Curve (*e*) corresponds with the curve in Fig. 2.

found to lie approximately on a straight line, except in the case of the dose of 1·28 units, which falls completely out of series. This behaviour of the points may be interpreted as indicating that a logarithmic relation exists between degree of calcification and dose of vitamin D. In other words, when the doses of vitamin D are increased in geometrical series, the corresponding values for degree of calcification, as measured by ash content of the dried fat-extracted bones, advance in arithmetical series. This relation holds good until a point is reached with doses of vitamin D as large as 1·28 units, where calcification is limited by the insufficiency of phosphorus available in the diet. The relation also fails with the lowest and no dose, so that the graph, though a straight line for the major part of its course, is really S-shaped.

If the curves plotted in Fig. 3, representing the series derived from values for 20 animals on each dose, and its two subdivisions into a more and a less rachitic series, are similarly replotted, using the logarithms of the doses from 0·03 to 0·32 unit of vitamin D, the three sets of points lie approximately along three converging straight lines (Fig. 4, curves (*a*), (*b*), (*c*)), indicating that the constant increment in bone calcification, corresponding with the same multiple of dose, is less for the less rachitic series and greater for the more rachitic one.

If a similar curve (Fig. 4, curve (*d*)) is plotted for the small series of values for 8 animals each, on 4 different doses (0·05, 0·1, 0·2 and 2·0 units of vitamin D), described in Section I, Exp. 1 (see Table I), the points corresponding with the logarithms of the 3 lowest doses lie on yet another straight line, but the value for the logarithm of the high dose of 2 units, like that of 1·28 units in Fig. 4, curve (*e*), lies completely out of series.

The five series of points described above lie only approximately on five converging straight lines. The positions of these lines have been calculated according to the formula given by Fisher [1925, § 26]; when they are drawn and produced in the direction towards which they are converging, they tend to meet about a point (*A*) located by a percentage ash value of about 54, corresponding with the logarithm of a dose of about 5 units of vitamin D. One of the curves (Fig. 4, curve (*b*)), that for the least rachitic series, tends to cut the others rather than converge with them to a point, a state of affairs which appears to depend on the apparent aberrance of one ash value, that obtained for the dose of 0·05 unit; if this one value is omitted from the series in the calculation, the resulting line for curve (*e*) tends to converge with fair accuracy to the common point.

It is conceivable that this point of convergence represents the value for normal calcification for the bones of rats of the age used in this work (7–8 weeks at the end of experiment), which would be attained with increasing dose of vitamin D, if deficiency of phosphorus in the diet did not act as a limiting factor. Reference to the curve of Dutcher *et al.* shows that in their series the value for the ash in the dried extracted bones of rats of corresponding age on normal diet, was about 56 %, a value which tends to support the supposition. Further research is, however, required to establish that the point, thus obtained by extrapolation of the curves, really has this significance, and further investigation of the suggestion is contemplated. In any case, even if the point did represent the value for normal calcification, its position and indeed that of the curves themselves, could only be regarded as fixed for rats of a given age, since the percentage ash in the normal bone rises steadily, reaching a height of about 62 % at about 12 weeks old [Dutcher *et al.*, 1925].

The significance of the variation in susceptibility to rickets of the different litters of young rats is also discussed by Bourdillon and Bruce in the ensuing paper (p. 506). The superficially obvious interpretation is that the rats showing the lowest calcification are those which at the beginning of the experimental period have the lowest bodily reserve of vitamin D. We have no knowledge of

the way in which such reserves may be drawn upon, but it is hard to imagine any regular way in which they might be put out, which would yet preserve the logarithmic relation observed between dose and calcification, in different series of rats, showing different degrees of sensitivity to rickets.

It should not, however, be forgotten that the action of the rachitogenic diet is a decalcificatory one, and if we may take the value of 42 % obtained by Dutcher *et al.* for the ash in the dried extracted bone, as roughly representative of the normal value for the bones of young rats of the weight and age (40–45 g. and 3–4 weeks) at the beginning of the experiment of those used in the present series, we must regard all rats showing, at the end of the experiment, a percentage ash of less than 42, as animals in which an active process of decalcification of the bone has been going on. This makes it very hard to envisage what may be actually happening in the bone, though it is easy to conceive that the more rachitic the bone was becoming, the more economical might be the use made of any small amounts of vitamin D available.

There is no doubt also that differences in the amount of phosphorus available to the rat from any source, by altering the unbalanced Ca/P ratio (4/1) in the rachitogenic diet, would raise or lower the level of calcification on a given dose of vitamin D. Key and Morgan [1932] have shown that by lowering the ratio of calcium to phosphorus from 4/1 to 2/1 healing of rickets in the rat is induced equal to that brought about by a daily dose of 0·7 unit of vitamin D. There is, however, no evidence to show whether, when the ratio is altered in this way, the curve relating calcification to dose of vitamin D would still show the characteristics shown by the curves in this series. An examination of these possibilities is contemplated.

The fact, that when the effect is plotted against the logarithm of the doses the resultant graph, over the major part of its course, is a straight line, was noted also by Bourdillon *et al.* [1931] in constructing an X-ray scale for use with curative experiments, but since they were dealing with empirical numbers allotted to appearances in the bone, it could not be deduced that their curve possessed the fundamental significance, which emerges clearly from the present study. As they point out, the possibility could not be quite excluded that the logarithmic nature of the scale was due to an effect on the eye, in accordance with the Weber-Fechner law.

The existence of the logarithmic relation between dose and effect on calcification, coupled with the fact that it is preserved even when rats of different levels of sensitivity to rickets are used, so that the relation can be expressed by straight lines inclined at different angles and probably converging to a point, provides material which should be capable of application in the evaluation of unknown substances for vitamin D.

Application of the scale to the evaluation of unknown substances for vitamin D.

In applying the scale thus prepared to the estimation of the value of an unknown substance for vitamin D, the curves derived by plotting the percentage ash against either the dose (Fig. 3, curves *a*, *b*, *c*) or the logarithm of the dose (Fig. 4, curves (*a*), (*b*), (*c*)) may be used. The experimental procedure would be in either case the same; two groups of 8–10 young rats would receive suitable doses, 0·01 and 0·02 γ of irradiated ergosterol in a standard 0·01 % solution, the doses thus equalling 0·1 and 0·2 units of vitamin D, while two other groups of 8–10 rats, similar in litter constitution, would receive two doses of the unknown substance. The results for each dose would then be averaged. If the resulting values were such that the values of the percentage ash obtained for the doses of standard fell directly on one of the curves in Figs. 3 and 4, the values for the unknown substance could be read off against the dose or logarithm of the dose, on that particular curve, provided that the values for the unknown fell within the range of the curve. If they did not it would be necessary to repeat the experiment, selecting fresh doses of the unknown, until doses were arrived at which gave average values for the percentage ash, falling within the effective range of the curve.

It might, however, happen that the average values for the percentage ash given by the two doses of the standard did not fall on any of the curves already obtained; in that case it would be very much easier to use the curves in which the ash values are plotted against the logarithms of the doses, since these graphs are straight lines and knowledge of two points is sufficient to construct the whole graph.

The scale should not be used for doses of material having a value lower than the equivalent of 0·03 or higher than the equivalent of 0·32 or perhaps 0·64 unit of vitamin D.

If a sufficiently definite position could be ascribed to the point (*A* in Fig. 4) of convergence of the various straight lines correlating the ash values with the logarithms of the doses, it would be possible, with the knowledge of the position of this point and of that of the average ash value derived from 8–10 rats on a single dose of the standard, to construct the whole graph for rats of that given degree of sensitivity. It is improbable, however, that the position of this point can be more than approximately determined.

No attempt has so far been made to evaluate any unknown substance against the scale and instances cannot be found in the literature where groups of animals as large as 8–10 have received serially graded doses of irradiated ergosterol, yielding results which could be compared with those in the present paper. Schultzer [1931], it is true, gives average results from ashing the bones of groups of 9 rats, receiving doses of 0, 1, 2, 3, 5, 10, 20 and 40 mg. of cod-liver oil. The average ash percentage in this series does not show any increase for doses above 3 mg., so that doses of 1, 2 and 3 mg. are the only ones

available for comparison. The percentage ash plotted against the logarithms of these doses gives points which are far from being on a straight line. The ash is, however, calculated as percentage ash in the fresh bone, and when thus calculated the values are subject to even greater irregularities than when calculated as percentage ash in the dry fat-extracted bone. Also his experimental groups, on the various doses, are not uniform in litter composition and unless such uniformity is very strictly preserved there would appear to be little hope of a smooth result, since variation between litters in their sensitivity to rickets is so great.

Summary.

1. The construction is described of a scale correlating doses of a standard solution of irradiated ergosterol (international standard for vitamin D), administered prophylactically, with the percentage ash in the bones of young rats on a rachitogenic diet.

2. The groups of animals on the different doses had the same litter composition, and, when 10–20 animals were used for each dose, smooth curves were obtained.

3. When the values for the percentage ash thus found were plotted against the logarithms of the doses (with the exception of the lowest and the highest) a linear relationship between the two variables was revealed, showing that as the doses were increased in geometrical progression the calcification increments advanced in an arithmetical series.

4. The whole series of 20 litters of rats could be divided into a more and a less rachitic series of 10 litters each. When the values for the percentage ash for these two series were similarly plotted against the logarithms of the doses, the logarithmic relation was preserved, but the values for the percentage ash, though advancing in arithmetical series, did so by smaller increments in the case of the less rachitic series, so that the straight lines in the graph tended to converge.

5. The application of the scales thus plotted to the evaluation of unknown substances for vitamin D is discussed.

The work here described was begun in connection with the inquiry, initiated by the Medical Research Council, into the stability of standard solutions of vitamin D and was further pursued at the request of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council, the assistance being financed by the Medical Research Council. The writers are, therefore, indebted to a large number of people for help and advice of various kinds, but particularly to Dr Burn who suggested the construction of the scale, to Sir Charles Martin, to Dr Chick and to Dr Coward.

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LXI. THE DETERMINATION OF VITAMIN D.
II. A COMPARISON OF RADIOGRAPHY AND BONE ANALYSIS IN THE ESTIMATION OF VITAMIN D.

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Through the friendly co-operation of Miss Hume and Miss Gaffikin, and of Dr Zilva, Miss Crawford and Miss Perry, we have been afforded the opportunity of radiographing the 180 rats referred to in the preceding paper (this *Journal*, p. 488) and 200 other rats, of which the bones were analysed by Dr Zilva and his co-workers at the Lister Institute. Sufficient data are thus available for a rough comparison of the radiographic and analytical methods, and for a study of the errors in both methods.

Technique.

Analytical method. The diet and analysis of Miss Hume's rats have been described in the preceding paper. The treatment of Dr Zilva's rats was on similar lines, except that the doses of vitamin were given in the form of natural butters or oils, of which the activity was being tested for purposes unconnected with this paper. Dr Zilva's results are expressed as the A/R ratio [Chick *et al.*, 1926], *i.e.* the ratio: weight of ash/(weight of dried fat-free bone *minus* weight of ash). The results of Miss Hume's analyses are here expressed as the percentages of ash in the dried extracted bones.

Radiography. The animals were killed by coal gas, and one knee-joint of each rat was photographed by X-rays from a hot cathode tube (dental type), using 4 amp. filament current, and 12 milliamp. high tension current at about 35,000 volts, from transformers. An exposure of 5 secs. was used, with a distance from plate to cathode of 16 inches.

A. Radiographic method (with prophylactic dosing).

Construction of radiographic scale.

A scale for prophylactic dosing was constructed on lines similar to those previously used for a curative radiographic scale. This was described in a report [Bourdillon *et al.*, 1931], which also contained full details of the methods of estimating errors that are used in this paper. A curative scale has also been

8 7 6 5

4 3 2 1

Enlarged × 2.

8 7 6 5 4 3 2 1

Natural size.

Radiographic scale for prophylactic dosing.

described by Everse and van Niekerk [1931]. For the prophylactic scale a series of photographs of developing rickets was selected, from rats used for testing vitamin D, such that each photograph showed an easily visible difference from the preceding member of the series. These are shown in Plate I, both natural size and enlarged to two diameters. It was found advisable to select only 8 stages, instead of the 12 that were used in the curative scale, since fine distinctions proved unreliable in the lower half of the scale. As with the curative scale the photographs were selected without reference to dose, and there is therefore no reason to expect any uniform relation between dose and resulting scale number. When matching photographs it is useful to recall the following criteria, which were used in distinguishing the various numbers.

Scale No. 8. Healthy bone, with no appreciable blurring of the top of the metaphysis.

Scale No. 7. Definite blurring at top of metaphysis, but only for a very short length.

Scale No. 6. Top of metaphysis blurred for appreciable distance (usually a small gap of uncalcified tissue between shaft and epiphysis).

Scale No. 5. Blurring extends to 1 mm. or more, and the bone has no definite edge to it. In some specimens the blurred area is less deep, but there is a very narrow gap of uncalcified tissue across the bone.

Scale No. 4. Definite narrow gap of uncalcified tissue right across the bone.

Scale No. 3. Uncalcified zone about 1 mm. wide.

Scale No. 2. Uncalcified zone wider, but still traces of calcification at lowest portion of new tissue.

Scale No. 1. Full rickets. Gap 1·5 mm. or more, without visible calcification.

In the earlier stages of this work a rough "verbal" scale was constructed, based on criteria similar to the above, but distinguishing 12 different stages, and used without scale photographs. Experience showed that variations in the rate of growth rendered the distinction of fine differences in the lower half of this scale rather unsatisfactory, and hence the number of divisions was reduced to 8 when making the photographic scale. However, the errors of result found with the rather crude "verbal" scale were of the same order as those of the photographic scale, and some of the results obtained by its use are quoted below. They show that it is possible to get very fair results without a photographic scale, but we do not recommend this method, as the subjective element is much larger than that involved in matching a photographic scale.

Relation between dose and effect.

Table I shows the results obtained by applying this scale to Miss Hume's rats. The litters are arranged in order of the mean ash percentage of the litter after dosing, beginning with the lowest. These data are illustrated in Fig. 1, which shows the curves obtained by plotting the logarithm of the dose

of vitamin against the radiographic scale number. The central curve No. 2 shows the mean results, for all litters, each point representing 20 rats. Curves 1 and 3 show the results for the groups of 7 litters that showed the highest and lowest ash percentages respectively.

Table I. *Radiographic scale readings of rats' knee-joints after prophylactic dosing.*

	Dose (in International Units)									Mean ash percentage for each litter for doses 0 to 0·32 only
	0	0·01	0·03	0·05	0·1	0·2	0·32	0·64	1·28	
Litter No.	Scale readings									
1867	1	3	1	1	3·5	5	5	—	—	31·36
1869	2·5	4	2·5	2·5	3·5	5·5	7	—	—	35·66
1828	4	4	4	6·5	6	6	8	—	—	37·01
2057	2	1	3	5	5	6	8	7·5	7·5	37·26
1834	5	5·5	4·5	5·5	6	7	7	—	—	38·39
2060	2	1·5	5	5	5	7	8	8	8	38·87
1827	5	4	7	5	6	6	8	—	—	38·97
Mean of 7 lowest litters	3·07	3·29	3·86	4·36	5·0	6·07	7·29	—	—	36·79
1854	2	4	4	5	6	7	6	—	—	39·76
1875	2	5	5	5·5	5·5	8	6	—	—	40·53
1914	5	5	5	5·5	6	6	7	8	8	41·84
1826	5	4	5·5	5	6	6	8	—	—	41·97
2095	1	2	3·5	4	4	5·5	6·5	8	7	42·14
1991	4	5	6	5·5	5	5·5	7·5	7·5	7·5	42·74
1915	4	7	4·5	5	6	7	6	7	7·5	43·01
1917	5	4	4	5·5	6	6	8	7	7·5	43·11
1988	3	5	6	5	5	5·5	7	6·5	7	43·13
1965	5	5	6	5	6	5·5	7	8	8	43·56
1943	5	5	5	4·5	5	6	7	8	7·5	44·06
1963	5	5	5	5	6	8	8	8	8	44·66
1954	5	5	6	6	7·5	7	7	8	7	48·61
Mean of 7 highest litters	4·57	5·14	5·21	5·14	5·93	6·45	7·14	7·5	7·5	44·31
Mean for all litters	3·62	4·2	4·62	4·85	5·42	6·27	7·1	7·62	7·54	40·83

These curves diverge with decreasing dose to an important extent. For doses of less than 0·05 (International) unit the highest litters give a nearly horizontal curve, suggesting either that serious amounts of vitamin were present in the diet or stored in the animals' bodies, or else that some other factor influenced conditions in such a way as to render the development of full rickets impossible. This point is discussed later.

The convergence and approach to parallelism of the upper portion of the three curves is noteworthy (especially when contrasted with the results of bone analysis shown in Fig. 2). It suggests that the dose of vitamin required to prevent the development of faint traces of rickets is much more constant than the doses required to prevent the development of full rickets, and may be more constant than those needed for the cure of developed rickets, in different litters. If this were the case, it would account for the statements of certain

workers, who use as their criterion "complete protection" from radiographic rickets, instead of cure or bone analysis, that they can obtain a stock of practically constant sensitivity without great difficulty.

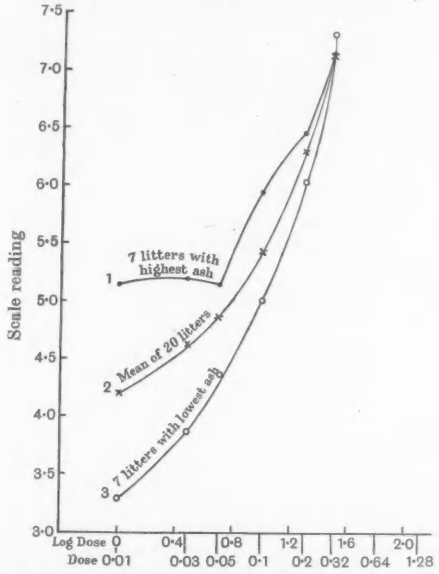

Fig. 1. Radiographic scale. Relation between dose and effect[1].

Evaluation of results from radiographic scale.

Owing to the curvature of the centre line in Fig. 1, it is more satisfactory to evaluate results by graphical methods than by an equation. If rats are dosed in pairs of litter-mates on standard and unknown solutions, it would be strictly correct to evaluate each pair separately. But in practice, if the doses used do not cover a wide range, it is sufficiently accurate, and much less laborious, to use the mean scale reading of the rats on standard solution, and the mean reading for the rats on corresponding doses of the test solution. The logarithms of the doses corresponding to these two mean values are then read off from the curve, and the antilogarithm of the difference between the two logarithms equals the ratio of the strengths of the standard and unknown solutions.

Since, however, the slope of the curve varies appreciably with the sensitivity of the stock, it is not desirable to use one curve for all tests, unless the stock is fairly constant; but for doses above 0·05 unit, for which alone this method is suitable, this variation in slope is not very great, and the plotting

[1] For convenience the unit of dose used when plotting logarithms was 1×10^{-6} mg. of irradiated ergosterol. This is one-hundredth of the International unit which is used elsewhere throughout this paper.

of a separate curve for each test is only necessary when the doses of standard and unknown solutions have been chosen so as to give mean scale readings of widely different values. Whenever possible the doses should be chosen so as to give approximately equal quantities of vitamin from the standard and the test solutions. When this has been done, the curve is only used to apply a small correction, and minor variations in slope are then of small importance.

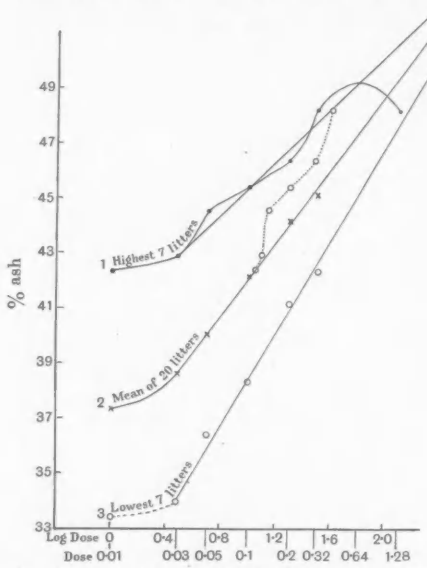

Fig. 2. Bone analysis. Relation between dose and effect.

Errors in radiographic estimation.

Terminology. In order to compare the errors of different methods of estimation of a vitamin, it is desirable to express all errors in terms of dosage. This can be done whenever the relation between dose and effect is known. It is then convenient to express any error as the ratio "apparent dose"/"true dose," or as the percentage error in estimation of dose which would be caused by the error in question. Thus an error of + 0·5 scale division in the centre portion of the radiographic scale can be seen from the curve in Fig. 1 to correspond to a rise of 0·24 in the logarithm of the dose. Since the antilogarithm of 0·24 is 1·738, this error equals a dose ratio "apparent dose"/"true dose" of 1·74, or an error of + 74 %. It should be noted that a negative error, such as − 1 scale division, gives a dose ratio which is the reciprocal of the value for the same positive error (*e.g.* 1/1·74): but when expressed as a percentage, a negative error has a smaller numerical value than a positive error. Thus an error of + 100 % corresponds to a negative error of − 50 %. Throughout this paper we express as percentages positive errors only, and we obtain them by subtracting

1 from the dose ratio, and multiplying the remainder by 100. We also use the letter σ to mean standard deviation.

In practical testing it is useful to know the frequency with which any given error can be expected. We have therefore calculated (*a*) the "probable error," which corresponds to $2/3\sigma$, and is likely to be reached or exceeded once in every two tests, (*b*) the error corresponding to 2σ, which is likely to be reached or exceeded once in every 22 tests. We have usually estimated these figures on the assumption that each test is based on 20 pairs of rats (half on standard and half on test solution). The corresponding errors for any other numbers of pairs, say x, can be calculated by multiplying the standard deviation for the error in question by $2\sigma/\sqrt{x}$, or $2/3\sigma \div \sqrt{x}$, and translating the product into terms of dose ratio.

The chief errors in radiographic estimation may be grouped into four classes.

(1) Errors in estimation of degree of rickets expressed as scale numbers.
(2) Errors in evaluation of the scale numbers in terms of dose.
(3) Errors due to variation between different litters in response to vitamin.
(4) Errors due to variation between litter-mates.

(1) *Errors in estimation of degree of rickets expressed as scale numbers.* These are termed the "scale-matching error." Among any batch of photographs, some will resemble the scale photographs so closely as to allow of no doubt in allotting the scale numbers to which they correspond. But other photographs will not resemble any scale photograph precisely, and variations of one-half, or even one division, may easily occur in matching. These errors were tested as follows.

One observer matched 196 photographs twice, with an interval between the observations. The following results were obtained:

Difference in scale divisions between first and second reading	Frequency of differences	Product: Error × frequency
0·0	114	0
±0·5	66	33
±1·0	16	16
		49

Average error 0·25 division; sum of + differences 20; sum of − differences 29.

The standard deviation of the differences is 0·407 scale division. This, converted into terms of dosage (using the value for doses between 0·1 and 0·2 unit), gives a probable error of 5·5 % on a test using 20 pairs of rats.

This value is high, and amounts to about half the total error observed in practice (*cp.* Table II). We suspect that the error of matching in practical work is lower than that observed in this test, since in practice the radiographs of litter-mates are matched in rapid succession, and this removes the likelihood of a change of judgment occurring during the process. With regard to the differences recorded in the above table, it should be noted that a considerable

proportion of differences of 0·5 unit and 1 unit is to be expected in matching to any scale.

2. *Errors in evaluation of the scale numbers in terms of dose.* This error becomes zero when doses of test solution are chosen so as to give results equal to those obtained by the standard solution. In practice this is rarely attained, but if the stock used for any particular test is fairly uniform, and if the mean dose ratio of test solution/standard solution is between 1·5 and 0·66, and is calculated from a curve plotted for the rats in question, the error should only be small. If, however, the test results in a dose ratio of test solution/standard > 3 or $< 0{\cdot}3$, we think there is a possibility of large error with any method of estimation of vitamin D.

3. *Errors due to variation in sensitivity of different litters.* Errors due to interlitter variation may be very serious, or be entirely obviated, according to the method of testing. If tests are conducted by dosing pairs of litter-mates with standard and unknown solution, interlitter variation can only cause negligible errors. If no standard solution is used, the errors may be very large, as in the example given later in this paper. A common practice is to mix 6 or 7 litters, and to divide doses of standard and unknown solutions as uniformly as possible between the various litters. This reduces the interlitter errors, but does not entirely obviate them.

4. *Errors due to intralitter variations in sensitivity.* The variation in the sensitivity of litter-mates is considerable, although much smaller than that between different litters. It is difficult to estimate directly, but the combined error of result due to this cause, and to the scale matching error, is easily estimated as follows. The scale readings are tabulated for a series of pairs of litter-mates given two different doses in a constant ratio. The standard deviation of the differences between the readings for the two doses is then calculated in scale divisions, and converted into terms of dosage by the use of a curve such as is shown in Fig. 1. The results of this procedure, when applied to the radiographic readings, are shown in Columns 2, 6 and 7 of Table II. The upper portion of the table shows the errors for Miss Hume's rats for each pair of doses. Each of these results is calculated from σ for 20 pairs of rats, except for the highest dose pair, for which only 12 pairs of rats were used. It will be noticed that the errors fall steadily, from an enormous value for the smallest doses, to a relatively small value for the highest doses. The excessive error for the small doses confirms the opinion, which would be formed from mere inspection of the figures in Table I of this paper and Table IV A of the preceding paper, that the methods are unsuitable for doses of less than 0·05 unit. There are two reasons for this. Firstly, in the lowest part of the radiographic scale a small error in scale number causes a very large error in apparent dose, and, secondly, the actual variations in response of litter-mates appear greater, whether judged by bone analysis or by radiography. This is shown by the high values of σ for small doses in Columns 2 and 4 of Table II. The range of dosage

over which the radiographic scale is reasonably satisfactory is 0·64 to 0·05 unit with the rats concerned, covering, therefore, a range of about 12:1. The range is limited at the lower end by undue errors in result, and at the higher end by the completeness of protection against rickets. Inspection of curve 3 of Fig. 1 suggests, however, that with a stock of rats showing high sensitivity to rickets, the useful dose range might increase to 24 to 1 or even more, owing to the increase in steepness and smoothness of the curve for such litters at small doses.

Table II. *Errors of result calculated from radiographic and analytical data for tests on 20 pairs of rats.*

A. Miss Hume's rats.

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8	Col. 9
	Deviations for difference between 2 doses				Errors by radiography with prophylactic scale. % error expected once		Errors by bone analysis. % error expected once	
	Radiography		Bone analysis					
Doses concerned (units)	σ (in scale divs.)	Mean difference in scale divs.	σ (in ash %)	Mean difference in ash %	In 2 tests	In 22 tests	In 2 tests	In 22 tests
0·01 and 0	1·268	0·573	3·175	1·27	—	—	—	—
0·03 „ 0·01	1·477	0·425	3·847	1·25	71	401	66	255
0·05 „ 0·03	1·030	0·225	2·736	1·53	43	145	15	53
0·10 „ 0·05	0·739	0·625	2·952	2·025	15	51	16	57
0·20 „ 0·10	0·914	0·80	2·313	2·035	12	41	13	43
0·64 „ 0·32	0·7672	0·375	2·628	1·10	22	82	14	50

B. Dr Zilva's rats (26 pairs).

Variable doses. Ratio constant at 3:1	1·541	2·312	A/R ratio 0·1036	Mean 0·1870	11·5	38·7	11·1	32

C. Curative radiographic method [Bourdillon *et al.*, 1931].

Variable doses. Ratio constant at 2:1	1·529	2·0		(1931)	8·2	26		
	1·458	2·0		(1932)	8	25		

The central section of Table II shows the errors for 26 pairs of Dr Zilva's rats, given varying doses of butters in the ratio of 3 to 1 for each pair of littermates. The results agree well with those from Miss Hume's rats, and show close concordance, whether calculated from the photographic X-ray scale, or from the "verbal" scale mentioned above.

At the foot of the table corresponding figures are introduced for comparison from the curative radiographic scale. In this series each figure refers to results from 1000 pairs of rats.

It is interesting to note that Bills *et al.* [1931] calculated by a different method from that used above that the probable error of a line test using 20 rats was 10 %. If this is multiplied by $\sqrt{2}$ to give the error for differences between pairs, it gives an error of 14 %, which is of the same order as the probable errors found above.

Frequency of errors.

The statement that an error of 2σ and $2/3\sigma$ would occur once in 22 tests, and once in 2 tests respectively, rests on the assumption that the frequency of errors of different magnitude follows the "normal curve" of error. Unfortunately, the data available are too few to allow of drawing a satisfactory frequency curve showing errors of different magnitudes. We have, therefore, counted the frequency of errors of or exceeding $2/3\sigma$ and 2σ in the difference table for Miss Hume's rats, to see how far the assumptions made in Table II were justified. In 100 differences the errors of or exceeding $2/3\sigma$ were 51 for the bone analysis, and 52 for the radiography, agreeing well with the expected number of 50. The errors of or exceeding 2σ were 5 for radiography and 8 for bone analysis, the expected number being 5. There is thus an abnormal proportion of large errors in the bone analysis data, but the numbers are too small to show how far this is significant.

Frequency of errors exceeding 100 % or other given ratio. A rough estimate of the accuracy of any method can be given by noting the frequency of "reversals" for pairs of doses in any given ratio, *i.e.* of instances when the rat on the lower dose gives a higher result than a litter-mate on the higher dose. Table III shows the frequency of such cases in these results.

Table III.

Observer	Doses concerned Units	Dose ratio	Reversals per 100 pairs: Bone analysis	Reversals per 100 pairs: X-ray scale
Hume	0·03 and 0·01	3:1	35	20
,,	0·10 ,, 0·05	2:1	30	10
,,	0·20 ,, 0·10	2:1	10	10
,,	0·64 ,, 0·32	2:1	25	25
Zilva	Various	3:1	4	4
Hume	0·20 and 0·05	4:1	10	5

For comparison it may be mentioned that the frequency of reversals over a dose ratio of 2:1 with the curative radiographic scale, at the Institute for Medical Research, was found to be 99 in 2000 pairs, *i.e.* 4·55 per 100 pairs. While of interest as a rough test, this type of frequency table is too liable to errors of sampling to be a useful guide for comparison of different methods, except when dealing with many hundreds of pairs.

B. Bone analysis.

Relation between dose and effect.

Fig. 2 shows the results obtained by plotting the ash percentage in the case of Miss Hume's rats against the logarithm of the dose. The centre curve shows the mean results for 20 litters, the upper and lower curves show the mean results for the 7 litters yielding respectively the highest and lowest calcium percentages. The graph is of interest for four reasons.

1. The two lower curves are approximately straight over a considerable range of dose (from 0·03 to 0·32 or 0·64 International Units), and the upper

curve, although less regular, has a general course which suggests that it would be linear over the same range, were it not for random errors in the small number of rats represented (7 per point).

2. The three straight lines that can be drawn through these data converge to a point, which represents a calcium percentage of 55·2 and a dose of 10 units. However, increase of dosage of vitamin beyond 0·64 unit does not cause a rise in ash percentage, but even appears to cause a slight fall, as can be seen from Table IVA of the preceding paper. This fall is not confined to the litters showing highest average calcium, but is also seen in the litters showing low average calcium, such as Nos. 2057 and 2060, although in these litters doses of 64 mg. give results much below the highest figures reached in the series. A similar fall with increasing dose was found by Schultzer [1931].

These facts strongly suggest that the variation in response to vitamin shown by different litters is caused, not by storage of vitamin, or by traces of vitamin present in the diet at different seasons, but by a second variable of different nature, possibly some other constituent of the diet. This view is supported by the dotted curve in Fig. 2. This curve shows the results of assuming that each rat in the 7 litters showing high ash percentage had contained stored vitamin equivalent to a dose of 0·1 unit per day. Thus doses recorded as 0·01, 0·03, 0·05, 0·1, 0·2 and 0·32 units are plotted as 0·11, 0·13, 0·15, 0·20, 0·30 and 0·42 units respectively. Although this brings the lowest point on the curve to the centre straight line, the rest of the curve does not follow this line at all, but approaches the upper line.

3. The results are widely different from the X-ray curves, which converge to a point within the diagram corresponding to a dose of 0·3 unit. This is clear evidence that the physiological changes detected by bone analysis and by radiography are not equally affected by the different variables which control the response to vitamin in rats.

4. The three curves lie at widely different heights above the base line, corresponding to great differences in sensitivity of the various litters. Thus the 7 litters in curve 1 gave the same ash percentage with a dose of 0·01 unit, as did the 7 litters of curve 3 after a dose 32 times as great. This shows that, in testing with a stock of this range of variation, failure to use a standard solution might cause errors of over 1000 %.

Evaluation of results of test.

The graphical method of doing this has been discussed in the preceding paper, and is convenient for most purposes. Although the slope of the lines varies too greatly to allow of the use of the simple equation given for the logarithmic curative scale in our previous report, the following general equation may be used for evaluating results when a non-graphical method is desired:

$$\text{Log } D_2/D_1 = \frac{(n_2 - n_1)\log 2}{x}$$

where D_2 and D_1 are doses which produce ash percentages of n_2 and n_1, and x is the increase in ash percentage caused by doubling the dose of vitamin. This equation may be more conveniently expressed as Dose ratio = Antilog (ash difference $\times$ 0·3010/x). In Fig. 2 the values of x are 1·4, 2·0 and 2·4 for the upper, middle and lower lines respectively.

Errors in estimation by bone analysis.

These errors are closely similar to the errors discussed above, except that the error of analysis is substituted for the scale-matching error, and that the errors due to variations between different rats may be of different magnitude in the two methods, since the physiological changes used for estimation are not identical.

1. *Error of analysis.* There is no simple method of measuring this, but an estimate can be obtained by comparing the results for right and left legs described in the preceding paper. As there explained, this result shows the sum of the error of analysis and of any true asymmetry that may be present, and can therefore only be regarded as a maximal value for the error of analysis. The standard deviation for this difference is 0·5833, expressed in terms of ash percentage. When converted into terms of dosage this gives a probable error of 3 % in tests based on 20 pairs. The error is thus appreciable, but is only about 1/5 of the error of result shown in Table II, and about one-half of the scale-matching error found for the prophylactic radiographic scale.

The following test makes it seem probable that the asymmetry in calcification is very small, and that the chief part of the above errors is due to errors of analysis. The right and left legs of about 80 rats were compared by separate analyses and by radiography. In order to avoid any possibility of bias, the right and left legs were matched against the scale at different times, although owing to the different appearance of left legs from the right legs of which the scale is composed, this procedure involved a rather large scale-matching error. It was found that of the 7 pairs in which the ash percentage of the two legs appeared to show a difference of 1 % or more, the radiographic scale numbers were identical in six pairs, and only showed a difference of 0·5 division in the seventh. Of the 16 pairs in which the radiographic scale divisions appeared to show a difference of 1 or 1·5 divisions between the two legs, only 3 showed a difference in ash percentage larger than 0·6, and these were in the opposite direction to the difference in scale reading. Further, in the 9 cases showing a higher scale number in the left leg than in the right, the average ash percentage was lower in the left leg than in the right. This complete absence of correlation between radiography and bone analysis when applied to the differences between right and left legs, is strong evidence that the apparent differences are due to errors of analysis and of scale-matching rather than to true asymmetry. These results differ from those of Adams and McCollum [1928] who found evidence of appreciable asymmetry with bone analysis after curative dosing.

2. *Errors of result* (*including errors due to intralitter variation*). As with the

radiographic method, the combined effect of errors of analysis, and errors due to variation between litter-mates, can be obtained by calculating the deviations for pairs of litter-mates that have been given different doses of the same solution. The results of this procedure are given in Columns 4, 5, 8 and 9 of Table II. They indicate that the method is satisfactory for doses between 0·64 and 0·03 unit, but too erratic for smaller doses.

An alternative method of estimating errors of result is to calculate the deviations from the mean for a group of rats that have received the same dose of vitamin. In order to get a figure comparable with the standard deviation for differences between pairs the figure thus obtained must be multiplied by $\sqrt{2}$.

The series of Dr Zilva's rats contains 16 groups, each of 6 or 8 rats from a group of litters of approximately the same date, that have been fed on one dose for each group. The standard deviations of the differences for each rat from the mean for its group were calculated for each group separately from the formula $\sigma^2 = \Sigma x^2/N - 1$, where x is the deviation for one rat from the mean for its group and N the number of rats in each group. The square roots of the mean of the squares of the 16 values of σ thus obtained were 0·089 for the A.R. ratio, and 2·286 for the X-ray readings on the "verbal scale." On multiplying by $\sqrt{2}$ to get a value for differences between pairs, and then converting into terms of dosage by using the relation between dose and effect found for the 26 pairs quoted in Table II, this gives a probable error for a test on 20 pairs of rats of 12 % when calculated from the A.R. ratio, and 12·5 %, if calculated from the X-ray scale. These figures slightly exceed those shown in Table II, as would be expected, since this method of calculation is less reliable than that used for Table II, the results being increased by variations in sensitivity between different litters. In the case of Dr Zilva's rats, the litters receiving any one dose were of approximately the same date, and the interlitter error was small. On the other hand, the application of this method of calculation to Miss Hume's series leads to excessive estimates of error, as would be expected, since the litters including rats on any one dose varied greatly in sensitivity at different periods of the year.

C. Comparison of some different methods of estimation of vitamin D.

The above data afford a basis for making some comparisons between bone analysis and radiography, and, when compared with data already published, between prophylactic and curative methods. Such comparisons are most easily made under separate headings, according to the method of dosing, and of estimating the effect.

(*a*) *Comparison of bone analysis* versus *use of radiographic scale, with prophylactic dosing.* The following conclusions appear justified.

1. The accuracy of estimation of the particular effect measured is greater with bone analysis than with radiography (ratio of errors about 1 to 2), and

the small subjective element which is introduced into radiography by scale-matching is almost absent from the method of bone analysis.

2. The range of doses, and sensitivity of stock, over which satisfactory measurement of effect is possible is greater with bone analysis than with radiography (in the ratio of about 2 to 1). This is of appreciable advantage in practical testing.

3. The initial cost of (new) radiographic outfit is about £100, whereas bone analysis needs little equipment.

4. The error of result observed in tests of doses of known ratio appears approximately equal in the two methods over the more limited range of doses for which the radiographic method is suitable. This suggests that the metaphyseal changes in calcification are more strictly proportional to dose of vitamin than are the changes in the rest of the bone. The increased accuracy of analysis over scale-matching appears to be largely counteracted by this factor.

5. The chief source of error in both methods appears to be variations in the sensitivity of litter-mates. This error is reduced in proportion to the square root of the number of animals used for each test. Hence it is desirable to use as many animals as possible, and therefore the time and labour required become important factors, not merely in the cost of testing, but in the accuracy obtainable in routine tests. In this respect the speed of the radiographic estimation gives it a great advantage over bone analysis. The time required for radiography is about 2 minutes per rat, including killing and checking numbers. The scale-matching takes rather less than 1 minute per rat, including calculation of results. The development and washing of plates takes an appreciable time, but can easily be taught to unskilled boys.

(*b*) *Comparison of bone analysis with radiography, using curative dosing in both cases.* Owing to the irregular results obtained with bone analysis [*cf.* Schultzer, 1931; Hess *et al.*, 1931] when using curative dosing, there can be little doubt that radiography is preferable in this instance.

(*c*) *Comparison of bone analysis, using prophylaxis,* versus *radiography, using curative dosing.* In this comparison the following conclusions seem warranted.

1. For tests involving any given number of pairs of rats, there is not a great difference in accuracy between the two methods. The deviations observed in the prophylactic bone analysis series on 300 rats are from 50 to 100 % larger than those observed previously with the curative radiographic method but the difference is not large enough to be of certain significance on only 300 rats.

2. For routine estimation of vitamin D on any extensive scale the radiographic method is preferable, since the decreased labour involved allows of the employment of a larger number of rats, with consequent increase in accuracy.

(*d*) *Prophylactic dosing, radiographic method. Comparison of scale* versus *method based on "characteristic curve."* We have elsewhere [Bourdillon *et al.*, 1931] discussed methods based on a determination of the percentage of rats which, when given a uniform dose, reach any given standard of rickets. Such

methods are widely used on the continent [Holtz *et al.*, 1931], and are in effect based on principles similar to those discussed by Trevan [1927] and illustrated by "characteristic" or frequency curves. We concluded that, when using curative dosing, the use of a radiographic scale was preferable to the use of a "characteristic curve." However, when using prophylactic dosing the advantages of the characteristic (or frequency) curve became much more obvious. Consideration of the curve in Fig. 1 shows that the radiographic scale is most sensitive and most reliable at the upper end, *i.e.* that rats which are just developing the first trace of rickets show a more certain response to a given change in dose of vitamin than do rats with well developed rickets. Further, the range of dose over which the prophylactic scale is useful is limited. In view of this we think it an open question whether, if prophylactic dosing is used with radiography, it is better to use a scale, as above, or merely to determine the percentage of rats which, on a given dose, develop any visible trace of rickets, and to apply small corrections by a characteristic curve.

(*e*) *Comparison of prophylactic dosing* versus *curative dosing, using radiography for measurement of effect.* The prophylactic scale is inferior to the curative scale in that it covers a smaller range of dosage, and in the lower half of the scale is less accurate. The relation between effect and the logarithm of the dose is less simple, and varies at different parts of the scale. Further, variations in sensitivity of the stock affect the prophylactic scale and the relation between dose and effect more seriously than they do the curative scale. It is probably owing to these differences that the average observed error with the prophylactic scale is appreciably greater than with the curative scale.

Comparisons with results of other workers.

The relative merits of curative and prophylactic dosing have been discussed frequently. Thus Schultz [1929] and Poulsson and Lövenskiold [1928], have stressed the advantages of curative dosing, on the grounds that rats always vary, and that radiography before dosing is useful in detecting such variation and eliminating the grosser cases. We have found this a great advantage in our own curative work. On the other hand, many workers advocate prophylactic dosing as superior to curative, and some regard rats used in this way as of such unvarying sensitivity that they can serve as a reliable basis for definition of a vitamin unit, without reference to a standard [Scheunert and Schieblich, 1929; Holtz *et al.*, 1931]. However, Miss Hume's results show that prophylactic dosing does not necessarily prevent very wide variation in sensitivity of stock, and Holtz *et al.* [1931] admit that their own stock varies in sensitivity by more than 100 %.

It seems to us that the choice of method of dosage should depend on the method of detection of results. Thus there is little doubt that bone analysis is unsatisfactory with curative dosing, as is shown by Adams and McCollum [1928] and by the careful comparison made by Schultzer [1931]. This is

probably due to the fact that when a severely rickety animal is given vitamin D in moderate doses, calcium deposition begins at the metaphysis, and only affects the diaphysis later, and more irregularly [Hess *et al.*, 1931]. On the other hand, the line test, which has been studied in detail [Bills *et al.*, 1931; Dyer, 1931], is usually considered more satisfactory with curative dosing than with prophylaxis.

When radiography is used, the advantages of the two methods of dosing are more evenly balanced. We think that some of the arguments used against curative dosing with radiography are not unanswerable. Thus Schultzer states that it takes a longer time than prophylactic dosing. We have, however, found a total period of 28 days (14 preparative and 14 on dose, using a Steenbock diet) very satisfactory, although this period is identical with that used by most workers for prophylactic work. Again, it is possible that the irregular results found by Schultzer for curative radiography may depend to some extent on the rather short curative period of 7 days used by him. We have not ourselves been able to get satisfactory results in so short a time. The curative radiographic scale has also been criticised by Holtz *et al.* [1931] on the ground that a number of stages of healing must be distinguished. This is, however, a real merit in practice, since it allows of getting useful results from tests over a considerable range of dose, with a resultant decrease in labour when testing solutions of unknown concentration.

Summary.

1. A radiographic scale is described, showing various degrees of development of rickets, for use in estimation of vitamin D by measurement of its prophylactic effect.

2. The errors in estimation of the vitamin when using this scale and when using bone analysis are compared. While the error in determination of the resultant effect is considerably greater with the radiographic scale than with bone analysis, the total error of a test with any given number of rats appears roughly equal in the two methods provided that the doses of vitamin are confined to the more limited range of the radiographic method.

3. Owing to the decreased labour of the radiographic method it is easy to use a larger number of rats, and so to get greater accuracy than with bone analysis.

4. Study of the relation between dose and effect in the two methods suggests that the chief variations in the sensitivity of a stock of rats fed on a supposedly constant diet are due to some factor other than vitamin D.

5. The prophylactic radiographic scale is less satisfactory than the curative scale.

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LXII. THE DETERMINATION OF VITAMIN D.
III. THE STABILITY OF PREPARATIONS OF VITAMIN D.

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The stability of preparations of vitamin D is of importance, both for the commercial products now on the market, and especially for the standard solutions which are widely used in estimations of the activity of other preparations. At present the International Standard adopted at the London Conference in 1931 consists of a solution of irradiated ergosterol in olive oil, and although this is likely to be replaced within two or three years by preparations of the crystalline solid vitamin recently isolated, sub-standards of solutions in oil will probably continue in use for many years.

Although it is well known that cod-liver oil retains its antirachitic activity for a long time, little work has been done on the stability of artificially prepared vitamin D. Steenbock and Black [1925] found that irradiated olive oil lost all activity in nine months when freely exposed to laboratory air, but retained its activity when kept in a stoppered bottle for the same period. We are, however, aware of no detailed work on the stability of irradiated ergosterol in oily solutions, and we therefore undertook the following tests.

Owing to the considerable stability of vitamin D at room temperature, and the inaccuracy of current methods of estimation, tests of stability are difficult and laborious. Hence the tests described in this paper are admittedly incomplete. They do, however, show that preparations cannot be relied upon to retain their activity for a year at room temperature, and they give a rough guide to the probable keeping qualities of standard and other preparations. The majority of these tests were done at the request of the Vitamin D Sub-Committee of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council, in preparation for the International Conference of 1931.

Types of test employed.

A direct test of the stability of vitamin D can only be made by studying the effect of one sample on large numbers of animals at long intervals of time. However, this procedure is slow and inaccurate, owing to the varying sensitivity of successive stocks of animals to this vitamin. We have therefore used, in addition, the following indirect methods.

(*a*) *Comparison of the strength of similar preparations made at widely different dates.* If it were possible to use an identical procedure in the preparation from ergosterol of two samples of vitamin D made at widely different dates, a comparison of the two samples could be relied upon to show any change that had occurred in the first sample during the interval between the preparation of the two. Unfortunately, it is impossible to secure constant sources of ultra-violet radiation, and it would therefore be unsafe to assume that successive preparations were initially of exactly equal activity (except in so far as this could be controlled by biological tests). Hence tests of this type can only be relied upon to show gross changes (unless made on the pure isolated vitamin).

(*b*) *Determinations of the temperature coefficient of decomposition of the vitamin.* Tests at elevated temperatures show decomposition of an extent and velocity that can easily be measured. By plotting a curve of results at higher temperatures, and extrapolating the results to lower temperatures, it is possible to form a rough estimate of stability at 0°. This method has been used for insulin by Krogh and Hemmingsen [1928] and for oxytocin by Gaddum [1930].

(*c*) A further series of tests was made to determine the effect of adding to a standard solution large quantities of such impurities as might be expected to occur in small quantities in preparations of the vitamin. If impurities of a destructive type were likely to be present in standard solutions, no general tests of stability would be of value, and each solution would have to be tested separately. Fortunately our results show no such necessity.

Technique of tests.

The estimations of antirachitic activity were carried out by the radiographic method described in detail in a report already published [Bourdillon *et al.*, 1931]. Albino rats (Wistar strain) of age 3 to 4 weeks were given a modification of Steenbock's rachitogenic diet for 4 weeks. After 2 weeks on this diet, the rats were anaesthetised with ethyl chloride, and radiographs were taken of their right knees, in order to determine the degree of rickets produced. During the second 2 weeks, daily doses of vitamin D dissolved in oil were given by capillary pipettes into the animals' mouths. The animals were then killed, and again radiographed. The radiographs of the rats taken after dosing were compared with a standard scale of healing divided into 12 stages, and the results averaged as described in the report referred to. In the tests at varying temperatures half the rats in each litter were dosed with the heated solution, and half with some of the same solution that had been stored at $-4°$.

Estimation of errors in the biological tests.

An estimate of the accuracy of these tests was made by examining the standard deviations for 1000 of the rats used during the period covered by this work, and showed that, for tests based in each case on 20 pairs of rats, the

error corresponding to twice the standard deviation, *i.e.* the error expected to be reached or exceeded only once in 22 tests, was *plus* 25 % or *minus* 21 %. The probable error (or error expected once in 2 tests) is 8 %. These values are in close agreement with those calculated for earlier tests in the report referred to above.

Most of the tests described in this paper were done with approximately 20 pairs of rats for each test. In the cases where fewer rats were used the number is specified in the text or placed in brackets directly after the figure giving the result.

Nature of preparations subjected to test.

The following preparations were used.

(*a*) *Standards I to V*. These all consisted of 0·01 % solutions in olive oil of the irradiated ergosterol. These were prepared by dissolving ergosterol to 0·1 % solution in alcohol (Standard III) or ether (Standards I, II, IV and V) and exposing the solution in a closed vessel filled with nitrogen to unfiltered radiation from a mercury vapour lamp until 40 to 70 % of the ergosterol had been transformed. Standards I, II and III were irradiated in silica cells with plane sides enclosing a layer 1 cm. thick. Standard V was irradiated while flowing rapidly through a water-cooled spiral of silica tubing of bore 4 mm. After irradiation the solutions were evaporated at a low pressure, at or below 40°, to a small bulk. Some olive oil was then added, and evaporation continued in order to remove as much solvent as possible. The concentrated solution was then diluted with commercial olive oil. The dates of preparation were as follows: Standard I, February 1927; Standard II, April 1928; Standard III, February 1929; Standard IV, November 1928; Standard V, January 1931. The tests were mostly done during 1931. Standard III was part of the solution issued during 1930 as the Medical Research Council Standard, and during 1931 as the International Standard. Standard V is the solution now under International trial for adoption when the stock of Standard III becomes exhausted. Except where otherwise stated (as in Table I) all solutions were kept in stoppered bottles at about − 4° from preparation until the date of test.

The solid preparations referred to as "impure" or purified crystalline vitamin D are described in a paper previously published [Askew *et al.*, 1932]. They are now believed to have contained 50 %, and over 90 %, of the pure vitamin respectively.

Direct tests of one solution at different periods.

For this test samples of Standard IV were withdrawn from cold store every 2 or 3 weeks during a period of 21 months, and used as a standard during routine testing of vitamin D. If the general sensitivity of the rat stock had remained constant, the average degrees of healing of rickets produced during successive periods would have served as a measure of the stability of the

standard. However, in spite of careful control of the diet of the test rats, and that of their parents, the stock showed a large and irregular variation in sensitivity. The effects of this variation were partly, but only partly, obviated by selecting for this test only rats which had shown a full and satisfactory development of rickets (approximately 1·6 mm. of wholly uncalcified space at the head of the tibia) before dosing with vitamin began. Fig. 1 shows the results for successive groups of 20 rats thus selected, all dosed with one solution during 1929 and 1930. The abscissae show the dates. The ordinates show the average degree of healing per group of 20 rats, expressed on the right of the figure as scale numbers, and on the left as numbers proportional to the apparent activity of the solutions, taking that of the initial activity for the larger dose as 100. The upper curve shows the results for doses of approximately one International Unit per day and the lower one for doses of half a unit. The graph shows irregular variations large enough to indicate the impossibility of

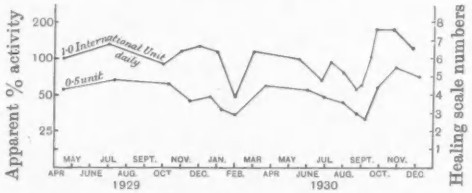

Fig. 1. Direct test of one solution during 21 months. Each point on the curves represents the mean result for 20 rats.

checking the stability of vitamin D by this method, unless a very long period of time is available, or unless a stock of rats of more constant sensitivity can be obtained. It can only be said that the average degree of healing produced at the end of the period of 21 months is slightly higher than that at the beginning, so that no great deterioration of the standard can have occurred.

We do not know the "errors of sampling" in this test, since our previous estimate refers to comparisons between litter-mates, and does not include the interlitter deviations which also have influence in this case. It is, however, clear from the similarity of the two curves, and from other similar tests [Bourdillon *et al.*, 1931], that the major variations are due to changes in the general sensitivity of the stock. We have as yet found it impossible to avoid such changes, in spite of attempts to keep the diets constant.

Indirect tests.

(a) Comparison of similar products prepared at long intervals of time.

An indirect test of stability was made by comparing the activities of Standards I, II and III. Although the conditions of preparation of these three solutions were not identical, there is reason to think that the initial activities of the three were roughly equal (*i.e.* probably within ± 50 %). The activities of these solutions were compared by Dr Coward and Miss Key at the Labora-

tory of the Pharmaceutical Society, and by Miss Hume and Miss Pickersgill at the Lister Institute, and one comparison was made in this laboratory. The results are given in Table I.

Table I.

Test No.	Date of test	Observer	Method of test	No. of pairs of rats used for test	Age of standard preparations in months I	II	III	Temp. at which standards had been stored	Result
1	Nov. 1929	Hume and Pickersgill	% ash in bones (prophylactic test)	12	—	19	10	0°	III=II
2	Apr. 1930	Hume and Pickersgill	% ash in bones (prophylactic test)	10 rats on II 20 rats on III	—	24	15	0°	III=II
3	Oct. 1929	Coward and Key	Line test (curative)	17	32	—	9	Room temp.	III/I=84/100
4	Oct. 1929	Coward and Key	% ash in bones (prophylactic test)	17	32	—	9	Room temp.	III=I
5	Apr. 1930	Coward and Key	Line test (curative)	24	—	24	15	Room temp.	III/II=100/50
6	Apr. 1930	Coward and Key	% ash in bones (prophylactic)	21	—	24	15	Room temp.	III/II=100/50
7	Feb. 1929	Webster	Radiography (curative test)	12	—	9	0	−4°	III=II

Tests 1, 2 and 7 show that no appreciable change occurred in Standard III during 9 months at 0° (unless it was initially stronger than II). Tests 3 and 4 show that Standard I did not deteriorate appreciably in 23 months at room temperature (unless it was initially stronger than III). Tests 5 and 6, however, suggest that when kept at room temperature, Standard II lost at least half its activity in 24 months.

(b) Tests of deterioration at different temperatures.

Consideration of the effects of temperature was first suggested by the experiment of Dr Coward given in Table I, in which one sample of Standard II showed a slow but definite deterioration at room temperature. It was not

Table II. *Percentage fall in activity of vitamin D preparations after exposure to different temperatures.*

0·01 % solution of solid products in olive oil.

Temp. °	Duration of heating (days)	Irradiated ergosterol in stoppered bottles: Standard III % fall	Standard III Velocity constant, k	Standard V % fall	Standard V k	Impure crystalline vitamin D in stoppered bottles % fall	Impure crystalline vitamin D in stoppered bottles k	Purified crystalline vitamin D (calciferol) in evacuated ampoules % fall	Purified crystalline vitamin D (calciferol) in evacuated ampoules k
Room temp. approx. 18	43	9	—	—	—	—	—	—	—
	83	0	—	—	—	—	—	—	—
	321	51	0·000965	—	—	—	—	—	—
40	66	33	0·002634	—	—	—	—	—	—
60	23	53	0·01430	—	—	—	—	—	—
	42	73	0·01354	—	—	—	—	—	—
80	10	56	0·03565	—	—	—	—	—	—
100	3	37	0·06690	—	—	—	—	—	—
	5	53	0·06558	25	0·025	27*	0·0273	43	0·0488
	10	75	0·06021	50	0·030†	51	0·031	59	0·0387

* 16 pairs. † 11 pairs.

certain whether the change in this sample was due to true instability at 20°, or to slow oxidation. It was, however, evident that a study of deterioration at higher temperatures was likely to give a rough guide to the stability at lower temperatures, and the series of tests shown in Table II was undertaken.

A further series of tests was made on solvent-free crystalline preparations with the following results. At room temperature a sample of impure crystalline vitamin showed no evidence of deterioration after 44 days in dry air or after 56 days *in vacuo*. At 150° a sample of purified crystalline vitamin (calciferol), in evacuated ampoules, gave the following results:

Duration of heating: hours	1	2	3·4	6	8
% fall in activity	25	45	63	83	90
k	3·00	3·11	3·05	3·08	3·00

Mean 3·05

These results are of interest in three respects.

(1) The decomposition of any one preparation at one temperature closely follows the unimolecular law, as is shown by the values of the velocity constant $k = 1/t\,(\log x - \log (a - x))$ where t = time in days, $a = 100$, and x = percentage of activity destroyed. These values are closely concordant at 150°, and fairly concordant for Standard III at 100°, and at 60°. The values for the other specimens at 100° are less concordant, but reasonably good, in view of the errors of biological testing. It follows that, if the reaction is bimolecular, the second reactant is present in considerable excess. In view of the low concentration of the vitamin such a possibility is not in itself improbable. However, the purest specimen of vitamin that we can obtain shows a rate of decomposition in a fused state *in vacuo* at 150° that does not differ greatly from the value expected from observations on a dilution of 1 in 10,000 in olive oil at lower temperatures, and this makes it very probable that the main reaction is truly unimolecular. Were it bimolecular the change of concentration should cause a very great change in velocity.

(2) The variation between different samples is appreciable, and exceeds the error to be expected from the biological tests. The causes of this variation are unknown, but variations in the different samples of olive oil, or in the relative proportions of the mixed irradiation products, may be responsible for the observed effect by causing subsidiary reactions. Further, the presence of varying volumes of air in the bottles below the stoppers might easily be of importance, since 0·07 cc. of air at 760 mm. and 0° would be sufficient to oxidise the whole of the vitamin in one 5 cc. bottle. To test this possibility a solution of purified vitamin was sealed in evacuated ampoules, after careful removal of dissolved gases by a rotary pump. However, this test showed a rate of decomposition closely similar to the other tests.

(3) The temperature coefficient of the velocity constants is of much interest. Fig. 2 shows the logarithm of the velocity constants for oily solutions plotted against the reciprocal of the absolute temperature. The mean result at each temperature is marked by a cross, the enclosed cross showing the result of test 5, Table I. The points deviate seriously from a straight line owing to

some unknown factor, possibly the side reactions already suggested as a possible cause of variations. The continuous line shows the most probable values for extrapolation, and suggests that at 0° 50 % of the vitamin would be destroyed in 3·6 years. The irregularity of the points allows of some latitude in extrapolation, but even by neglecting altogether one or other of the two lowest points, it is only possible to extend the half life period to $5\frac{1}{2}$ years or to

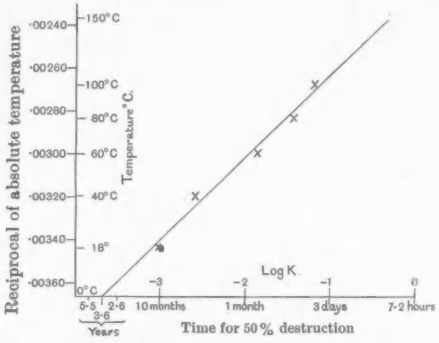

Fig. 2. Velocity of decomposition at different temperatures.

shorten it to 2·6 years. Hence 3·6 years can be taken as the probable time for 50 % destruction. This period is short enough to indicate grave difficulty in maintaining a permanent standard of the vitamin, and it is perhaps fortunate that a crystalline product which is believed to be pure, and of a reproducible activity, is now available. However, the extrapolation may be seriously at fault, owing to the fact that olive oil freezes at about 10°. There is some reason to think that decomposition of the vitamin occurs, or is accelerated, at the surface of contact with the glass containing vessels. Thus, *in vacuo* at 100° very small samples of irradiated ergosterol lose their activity much more quickly than larger samples. If decomposition is due to such a surface effect, or to oxidation, the enormous rise in viscosity of olive oil between 20° and 0° would stop the reaction almost entirely, and it is therefore not impossible that the vitamin is stable in oily solution for many years at 0°.

The value of μ calculated from the (Arrhenius) equation

$$\ln k_1 - \ln k_2 = \mu/2\,(1/T_2 - 1/T_1)$$

(where k_1 and k_2 are the velocity constants at absolute temperatures T_1 and T_2) varies rather widely, from 7750 to 17,340, at different parts of the curve. The mean value calculated from the graph is 11,800, which is of the usual order of magnitude. It may be noted that if log k is plotted directly against tempera ture a somewhat similar graph is obtained from which the mean temperature coefficient of the velocity constant per 10° is 1·74 between 20° and 100°.

(*c*) *Effects of impurities on stability.*

1. *Oxygen.* The effect of oxygen present during preparation of the vitamin is still uncertain. Tests show that oxidation products can accelerate decomposition *in vacuo* at 150°, and it is possible that the same is true for oily solutions at 18°. However, if such acceleration occurs, it is not excessive, since the Standards I and II mentioned above were prepared without complete exclusion of oxygen.

The effect of air on oily solutions of vitamin was tested as shown in Table III. These tests show that oxidation at room temperature is slow, but can be serious. In this connection it should be observed that in one of the cases of deterioration at room temperature already quoted (Table II) the bottle concerned was opened once only during the test, and in the other case (Table I, Standard II) the bottle was not opened at all until tested.

Table III. *Effect of air on Standard Solution III.*

Temperature	Duration of exposure days	No. of pairs of rats used	Conditions of exposure	Activity expressed as % of original
Room temp.	108	20	Exposed to air in a layer at the bottom of a beaker	75
,,	305	20	Exposed to air in a layer at the bottom of a beaker	Less than 10
,,	84	10	5 cc. in a bottle with stopper removed	107
−4°	50	11	5 cc. in 50 cc. stoppered bottle, containing air above solution	112

2. *Effect of addition of reagents which might possibly be present as contaminants.* These tests were made on 30 cc. samples of Standard V, kept at 40° for 29 days in full stoppered bottles. The results are only rough approximations, since the number of rats used, 3–5 pairs per test, was too small for accuracy. They suffice, however, to show whether gross deterioration did or did not occur.

Destruction of not less than 75 % of the original activity was caused by the addition (in each case to a sample of 30 cc.) of either (*a*) 0·2 cc. of a saturated solution of sulphur in benzene, or (*b*) 20 mg. of flowers of sulphur (arsenic-free) for 20 days, or (*c*) 0·4 cc. of concentrated sulphuric acid. On the other hand, no deterioration was detected after the addition of 0·6 cc. 25 % aqueous sulphuric acid; 1·9 cc. 40 % aqueous oleic acid; 0·2 cc. 0·880 ammonia solution; 0·7 cc. *N*/5 NaOH solution; 0·2 cc. 20 % aqueous KNO_3 solution; pieces of metallic silver, copper, lead, steel, and calcium; 0·1 cc. acetone or acetaldehyde; 0·1 cc. perhydrol (H_2O_2).

Similar tests, also at 40°, on samples of Standard V, diluted with two different brands of commercial "olive oil" bought in small bottles, showed no serious deterioration in the 29 days of the test, but a third sample showed an apparent loss of 37 % in activity, in a test based on 10 pairs of rats. This fall is within the margin of possible error for that number of rats, but is large

enough to be suspicious. In view of the commercial importance of oily solutions of the vitamin, tests with other oils than olive oil seem desirable.

The results of these experiments indicate that most of the inorganic or organic impurities likely to be present in small quantities in solutions of irradiated ergosterol cause no great reduction in stability, with the exception of sulphur. It appears important to avoid this impurity in such preparations, and the possible danger from the use of vulcanised rubber in tubing or stoppers should be noted.

SUMMARY.

Tests of the stability of vitamin D in solution in olive oil show that such solutions may deteriorate seriously if kept at room temperature for periods of a year or more. Deterioration at 0° has not been detected in the time available. Extrapolation from results at higher temperatures suggests that preparations at 0° may lose half their activity in about 3 years, but there is some reason to think that the solidification of the oil has a preservative effect, and that the stability at 0° may be greater than the experiments suggest.

The addition of various common substances likely to be present as impurities has no very great effect, with the exception of sulphur, which causes rapid deterioration.

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LXIII. THE SELECTIVE FERMENTATION OF GLUCOSE AND FRUCTOSE BY YEAST.

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Interest in the phenomena of selective fermentation of mixtures of sugars by yeast has been revived in the last decade, following the more precise measurements of Willstätter and Sobotka [1922]. The facts are briefly as follows.

(1) That separate solutions containing more than 1 % of glucose or fructose are fermented at equal rates by living yeast.

(2) That glucose and fructose in mixtures are fermented at unequal rates, glucose being fermented faster than fructose by most yeasts, including *S. cerevisiae*, while fructose is fermented faster than glucose by some yeasts, among which are Sauterne yeast, *S. pombe* and *S. exiguus*.

(3) That zymin generally exhibits selective properties similar to those of the yeast from which it is prepared, although the zymins obtained from *S. pombe* and *S. exiguus* show a selectivity opposite to that of the yeasts from which they are obtained [Fernbach, Schoen and Mori, 1928].

(4) That zymin prepared from brewer's yeast when acting on separate sugar solutions esterifies fructose more rapidly than glucose if a suitable concentration of inorganic phosphate is added.

Willstätter and Sobotka expressed their results in terms of a selectivity constant

$$K = \frac{\ln y_0 - \ln y}{\ln z_0 - \ln z},$$

where y_0 and z_0 represent the initial concentrations of glucose and fructose, and y and z represent sugar concentrations after a fermentation period t. K, therefore, is the ratio of the separate velocity constants calculated for unimolecular reactions, and its use appears to imply the assumption that the rate of fermentation of each separate sugar is proportional to its concentration at any time. The values of K obtained by Willstätter and Sobotka from different experiments showed good agreement.

Hopkins [1928], using the same formula as Willstätter and Sobotka, found $K = 1{\cdot}8$ for a top brewery yeast and $K = 2{\cdot}1$–$2{\cdot}8$ for a bottom yeast. In a later paper Hopkins [1931] determined the selectivity constants of brewer's yeast and of Sauterne yeast, and he also estimated the rates at which these two

yeasts ferment separate solutions containing glucose and fructose. These separate rates of fermentation are of particular interest since Hopkins found that, at concentrations of sugar below 1 %, brewer's yeast fermented glucose faster than fructose. The Sauterne yeast, on the other hand, fermented fructose faster than glucose at all concentrations of sugar tested, the effect being more marked in dilute sugar solutions. According to the ideas to be expressed in the present paper, the explanation of the phenomena of selective fermentation is to be found in the relative rates of fermentation of solutions containing low concentrations of the separate hexoses.

On the basis of the theory of Michaelis and Menten [1913] the relative rates of reaction of two substrates competing for the same enzyme can be expressed by the formula

$$\frac{V_1 K_2}{V_2 K_1} = \frac{\ln y_0 - \ln y}{\ln z_0 - \ln z},$$

where K_1 and K_2 are the Michaelis constants (expressed in concentrations) of the two substrates, and V_1 and V_2 the maximum velocities for each substrate alone [Haldane, 1930].

Since, as was shown by Slator [1908], the maximum rates of fermentation of glucose and fructose by brewer's yeast are equal, it is evident that the above equation is identical with that used by Willstätter and Sobotka to express selectivity. The selectivity constant thus represents the ratio of the affinities of the two sugars for the enzyme. From the figures given in Table IV by Hopkins [1931], the concentrations of sugar at which half the maximum velocity of fermentation is observed are 0·2 % and 0·4 % for glucose and fructose respectively. These figures yield a value of 2 for the selectivity constant; Hopkins obtained from mixed fermentations the value 2·3 at 31°.

The extent to which Hopkins's results on the rates of fermentation of separate sugar solutions agree with the Michaelis equation

$$v = \frac{VS}{K+S},$$

where V is the maximum velocity, v is the velocity at substrate concentration S, K is the Michaelis constant, can be tested by plotting the values of $\frac{1}{v}$ against $\frac{1}{S}$. Since the Michaelis equation may be transformed to $\frac{1}{v} = \frac{1}{V} + \frac{K}{V} \cdot \frac{1}{S}$, the figures plotted in this manner should yield a straight line. The agreement found is not sufficient to enable this method to be used to obtain values for the Michaelis constants more accurate than those obtained by direct observation of Hopkins's data. The determination of the rate of fermentation of low concentrations of sugar is, however, complicated by the autofermentation of yeast carbohydrate, and the amount of autofermentation is not independent of the sugar concentration. Fair agreement with Hopkins's experimental data is obtained by calculating the rates of fermentation at different sugar concentrations from the Michaelis equation using 0·2 % and 0·4 % as the values of the Michaelis constants for glucose and fructose respectively.

When using Sauterne yeast Hopkins observed that the rate of fermentation of glucose was less than that of fructose at all concentrations tested. In order, therefore, to calculate the selectivity constant from the Michaelis constants this difference in maximum velocities has to be taken into account. Analysis of Hopkins's data shows, however, that better agreement with the Michaelis equation is obtained if it is assumed that the maximum velocities of fermentation of glucose and fructose by Sauterne yeast are identical. This assumption can, to some extent, be justified since Hopkins's figures show that at 10·5 % glucose the velocity of fermentation was still increasing. Higher concentrations were not tested, but the theoretical maximum velocity is not likely to be achieved in practice on account of inhibition by hypertonic sugar solutions. Calculated on this basis Hopkins's data yield the values 2·5 % and 0·22 % for the Michaelis constants of glucose and fructose respectively, from which the calculated value of the selectivity constant is 0·09. Hopkins [1931, Table I] found the value 0·098 for Sauterne yeast at 31°. It is clear, therefore, that the phenomena of selective fermentation by living yeast can be satisfactorily explained by the Michaelis theory of enzyme affinity.

Selective fermentation by zymin.

In the above treatment of selective fermentation by living yeast no attempt has been made to ascribe the selection to any particular enzyme in the zymase complex. The most probable stage for selection seems to be during the reaction which results in the formation of hexosephosphate. A study of the apparently contradictory facts reported on selective fermentation by zymin yields some evidence for this hypothesis.

If the selection is due to the enzyme which catalyses the reaction between hexose and phosphate, it follows that the concentration of phosphates present should be taken into account. In this connection it should be noted that yeast-juice, dried yeast and zymin, when fermenting sugar, differ from living yeast in the manner of their response to added phosphate [Harden, 1932]. The hypothesis is now advanced that the selectivity shown by zymin when fermenting mixtures of glucose and fructose is controlled by the phosphate concentration.

Hopkins [1928] investigated the velocity of esterification of glucose and fructose by zymin (brewer's yeast) in the presence of varying concentrations of phosphates, and, in agreement with the results obtained by Harden and Young using yeast-juice, found that the maximum rate of esterification of fructose was greater than that of glucose, while the concentration of phosphates required to produce this maximum effect was greater for fructose than for glucose. The concentration of hexose in these experiments was 13 %. It is probable, therefore, that the affinity of the enzyme system for phosphate is smaller for the reaction catalysed between fructose and phosphate than for the reaction between glucose and phosphate. By applying Hopkins's figures for the maximum rates of esterification of separate sugar solutions to the Michaelis

equation it is found that the Michaelis constants are 0·05 *M* phosphate for the reaction with glucose and 0·12 *M* for the reaction with fructose, while the ratio

$$\frac{\text{Maximum rate of esterification of fructose}}{\text{Maximum rate of esterification of glucose}} = 1{\cdot}6.$$

These values are approximate ones, and the calculated maximum velocities are greater than those observed by Hopkins. Excluding the lowest phosphate concentration in the case of fructose and the higher concentrations in the case of glucose, the velocity figures given by Hopkins agree with the values calculated by use of the above constants.

A consideration of the values obtained for the Michaelis constants and for the ratio of the maximum velocities showed that, at low concentrations of phosphate (0·02 *M*), the rate of esterification of glucose should be greater than that of fructose, since at low concentrations of phosphate the greater affinity of the enzyme for glucose should outbalance the effect of the greater maximum velocity of the reaction with fructose. The effects on zymin of concentrations of phosphate of this order are not easy to measure, but they are of importance in the consideration of the phenomena of selective fermentation. Thus Hopkins [1928, Exp. 4] observed that zymin preferentially fermented glucose from a mixture of glucose and fructose. The solution fermented contained 10 g. zymin in 75 cc., and no phosphate was added. An estimate of the effective concentration of phosphate present in these experiments can be obtained from the data of Euler and Myrbäck [1923] who investigated the influence of phosphate concentration on the rate of fermentation of glucose by dried yeast. From their results these workers calculated that 1 g. of their dried yeast preparation contained 0·015 g. PO_4. Assuming the effective phosphate concentration of Hopkins's preparation to be of the same order, the phosphate concentration in his experiments would be about 0·02 *M*, under which conditions, according to the hypothesis developed above, selective fermentation of glucose is to be anticipated.

It is probable that a similar consideration of the various Michaelis constants concerned will explain the results of Fernbach, Schoen and Mori [1928] who found that zymins prepared from two yeasts exhibited selective fermentation opposite to that shown by the fresh yeasts, but in this case the interpretation is complicated owing to the use of a mixture of racemised sugars.

Discussion.

The degree of selectivity exhibited by any particular yeast is not constant, but depends on the cultural conditions to which the yeast has been subjected during growth. Thus Willstätter and Sobotka showed that the value of the selectivity constant of a yeast which preferred glucose to fructose could be lowered by cultivating the yeast in a medium containing fructose. This result was confirmed by Sobotka and Reiner [1930] who found that the preference of a Sauterne yeast for fructose was lowered by cultivating the yeast in

maltose. It thus appears that living yeast possesses the power of adapting itself to the medium in which it is propagated. Explained by the Michaelis theory, this type of adaptation must be considered as being due to an alteration in the affinity of the enzyme for the proffered substrate.

Evidence for the variation in the affinity of an enzyme for its substrate already exists in the results of Kuhn [1923] who showed that the affinity of yeast invertase for sucrose varied in different samples of *S. cerevisiae* examined, while Willstätter, Kuhn and Sobotka [1924] observed a similar lack of constancy in the affinity of yeast maltase for maltose and for α-glucosides. Since a variation of this type is observed with what are regarded as different races of *S. cerevisiae*, it is not surprising that the same race of yeast should, when grown under different conditions, exhibit differences in the affinity constants of its enzymes for particular substrates.

It is not suggested that the above treatment is more than an outline of the methods by which we may arrive at an understanding of the factors governing selective fermentation. A strictly mathematical treatment should include an analysis of the effect of the variation in concentration of one substrate on the affinity constant of the other substrate. The problem is, however, a biological one; selection by living yeast takes place within the cell, and our knowledge of the localisation and co-ordination of the enzyme systems in the cell, and of the methods by which the local concentrations of the substrates near the enzyme may be altered, is negligible. It is well known that the destruction of the organic whole, which occurs when the yeast cell is dried or macerated, injures the mechanism which controls the reaction between hexose and phosphate. It is, therefore, not surprising that with such dead preparations the degree of selectivity, which, in the living cell, is apparently controlled by the affinity and concentration relationships which exist between the enzyme and its competing substrates, should also be profoundly modified.

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